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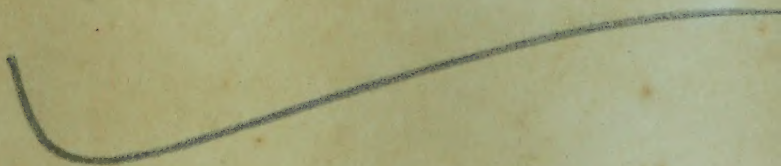


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The Chemistry of LIVING CELLS

by

HELEN R. DOWNES

*Professor of Chemistry,
Barnard College, Columbia University*



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Preface

This book is the outgrowth of a course in biochemistry which has been given at Barnard College for the past twenty years. The students have been senior majors in chemistry or in one of the biological sciences. The subject matter included in the book has been chosen not only to provide the material for a course in the fundamentals of biochemistry, but also to serve as a simple reference book for students whose primary interest is in zoölogy or botany or related fields. In practice it has always been found necessary to review the laws of solutions and the chemistry of the foodstuffs before attempting to deal with the complex transformations which take place in the cell.

The historical approach has been used where it seemed suitable in the belief that students should be introduced to the outstanding scientists in their field and encouraged to give them "a local habitation and a name." In this way students may begin to sense the nature of the great intellectual adventure which we call science, to understand how it progresses, and to appreciate the qualities which go toward the making of a scientist. Above all they may be led to meditate upon a truth which T. H. Huxley stated long ago: "... anyone who is practically acquainted with scientific work is aware that those who refuse to go beyond fact, rarely get as far as fact; and anyone who has studied the history of science knows that almost every great step therein has been made by ... the invention of hypotheses, which, though verifiable, often had very little foundation to start with; and, not infrequently, in spite of a long career of usefulness, turned out to be wholly erroneous in the long run."

As far as possible the subject matter has been presented in conjunction with specific experimental data, but since this is an introductory textbook, no attempt has been made to cite individual authorities for each statement. The references given at the ends of the chapters have been chosen with a view to helping the inexperienced student find his way into the subject. Recent review articles have been included not only for their full bibliographies, but because a logical and coherent summary of a mass of experimental data is often of great assistance to a beginner. The references given to original papers have been chosen either to emphasize work which has been of special significance or to bring the bibliographies up to date with respect to the main topics discussed in the text. The ma-

jority of the papers cited are in English; when references are given to German or French sources, the titles have been given in translation.

I am, as the figures in the book bear witness, greatly indebted to a number of people who not only have allowed me to reproduce material from their books and papers, but have couched their permissions in terms of friendliness and encouragement that went far beyond the call of duty. To all of these and to their publishers in England, Switzerland, Germany, Sweden, and here at home I tender my grateful thanks.

To two colleagues on the Barnard College Faculty I owe special debts of gratitude. Professor Victor Larsen of the Botany Department has done his best to save me from the consequences of my own ignorance of his science, and Professor Ingrith Deyrup of the Zoölogy Department, in the performance of a like service, not only has read and criticized portions of the manuscript but has taken time for many helpful discussions. The extent to which the book is sound zoölogically and botanically is a result of the friendly efforts of my colleagues; its shortcomings, alas, are my own.

I count myself especially fortunate in having had during the writing of this book the encouragement and wise advice of my former teacher, Professor Emeritus Marie Reimer of the Barnard Chemistry Department. She has read a large part of the manuscript and has made many valuable suggestions as to its organization and presentation. This present occasion for expressing my gratitude to her is but one in a long series, dating back to my own student days when her contagious enthusiasm first inspired in me the desire to become a chemist.

In such a book as this I have inevitably attempted to simplify and summarize material which is far outside the field of my own major interest. I shall therefore be especially grateful if readers will point out mistakes of fact or of interpretation.

HELEN R. DOWNES

New York
September, 1954

Abbreviations

Adv. in Carb. Chem.	Advances in Carbohydrate Chemistry
Adv. in Enzym.	Advances in Enzymology
Adv. in Prot. Chem.	Advances in Protein Chemistry
Am. J. Physiol.	American Journal of Physiology
Ann.	(Justus Liebig's) Annalen der Chemie
Ann. chim. phys.	Annales de chimie et de physique
Ann. Rev. Biochem.	Annual Review of Biochemistry
Arch. Biochem. Biophys.	Archives of Biochemistry and Biophysics
Arch. ges. Physiol.	Archiv für gesamte Physiologie
Beitr. chem. Physiol. u. Path.	Beiträge zur chemischen Physiologie und Pathologie
Ber.	Berichte der deutschen chemischen Gesellschaft
B.J.	Biochemical Journal (London)
Bull. soc. chim.	Bulletin de la société chimique de France
B.Z.	Biochemische Zeitschrift
Chem. and Eng. News	Chemical and Engineering News
Chem. Revs.	Chemical Reviews
Compt. rend.	Comptes rendus hebdomadaires des séances de l'académie des sciences, Paris
Fed. Proc.	Proceedings of Federation of American Societies for Experimental Biology
Helv. Chim. Acta	Helvetica Chimica Acta

Ind. and Eng. Chem., Anal. Ed.	Journal of Industrial and Engineering Chemistry, Analytical Edition
J.A.C.S.	Journal of the American Chemical Society
J.B.C.	Journal of Biological Chemistry
J. Colloid Sci.	Journal of Colloid Science
J.C.S. ¹	Journal of the Chemical Society (London)
J. Gen. Physiol.	Journal of General Physiology
Jour. Physiol.	Journal of Physiology (London)
Naturwiss.	Naturwissenschaften
Physiol. Revs.	Physiological Reviews
Proc. Natl. Acad. Sci. U.S.	Proceedings of the National Academy of Sciences of the United States of America
Proc. Roy. Soc.	Proceedings of the Royal Society (London) Series A. Mathematical and Physical Series B. Biological
Proc. Soc. Exp. Biol. Med.	Proceedings of the Society for Experimental Biology and Medicine
Trans. Faraday Soc.	Transactions of the Faraday Society
Z. angew. Chem.	Zeitschrift für angewandte Chemie
Z. physikal. Chem.	Zeitschrift für physikalische Chemie
Z. physiol. Chem.	Zeitschrift für physiologische Chemie (Hoppe-Seyler's)

¹ Recent numbers carry no volume number. The year of publication is given in parentheses.

Part I

Some Preliminary Considerations

Biochemical History and Literature

But this is certain; by how much one man has more experience of things past, than another; by so much also he is more Prudent and his expectations the seldomer faile him.

THOMAS HOBBS: *Leviathan* (1651)

Biochemistry is that branch of science which is concerned with the chemical events which take place in living tissues. It has existed as a separate discipline for less than one hundred years, but is rooted in that distant past, almost lost in the mists of prehistory, in which men first began to observe the world about them. Thus Babylonia contributed to science the beginnings of astronomy and of mensuration, and the Egyptians' skill in embalming bears witness to their knowledge of anatomy. Although these earliest-known civilizations have left few records of their own, it is clear from the writings of the early Greeks that the knowledge which they had accumulated was disseminated by traders and travelers throughout the eastern Mediterranean lands and became part of the Hellenic heritage. This heritage had been growing in a haphazard, empirical, and often accidental way for several millenia. But in the course of comparatively few years it was to be transmuted by the Greeks into a new way of living and thinking. It is not easy to define even superficially the Greek contribution. It was partly a rare clarity of mind. It was partly the first stirrings of the experimental method. But chiefly it was an attitude toward life and a belief in the fundamental importance of truth and beauty which worked together in the social structure of that small nation to foster a freedom of the human mind and spirit which is unique.

Athenian Science

The story of civilization in Greece and on the islands and mainland of Asia Minor can be traced back beyond 1500 B.C., but the earliest records of Greek biological science date from about the sixth century before Christ. By this time the great colonizing period was over, and people of the Greek race were established in city-states not only on the Greek mainland, but in southern France, in Italy and Sicily, around the Aegean and on its islands, and even on the shores of the Black Sea. Thus it happened that the two outstanding figures in Greek biological science although closely

associated with Athens were neither of them born in Greece itself. The physician Hippocrates¹ was born on the island of Cos and Aristotle's home was the colonial city of Stagira on the shore of Thrace.

Hippocrates, who was born about 460 B.C., lived for nearly one hundred years, traveling about the regions bordering the Aegean, teaching and practicing medicine. His ideas were not only far in advance of his own time, but of any intervening time until very recent ones. He taught first-hand observation of disease and patient checking of facts, and expressed clearly for the first time the fundamental faith on which all progress in science still depends, namely belief in an orderly universe in which effects follow inevitably from natural causes. For example, in the Hippocratic work dealing with the plague we find, "As for this disease called divine, surely it has its nature and causes as have other diseases." This was a far cry from the attitude toward disease which is characteristic of primitive people, that it results from the anger of the gods and can be cured by magic and incantation. It is of course impossible at this distance in time to know how many of the writings of the so-called "Hippocratic Collection" are truly the work of the great physician himself. But what is certain is that the medical learning which he collected and systematized was treasured by generations of students in what were called schools of Greek medicine though many of them were founded long after the fall of Greece. And although the learning of these schools crystallized into a stifling dogma during the Dark Ages, yet the spirit of modern medicine can be traced back in almost unbroken continuity to that springtime of learning in which Hippocrates lived and taught.

Aristotle (384-322 B.C.) as a young man was sent from his provincial home to study under Plato in Athens. Some years later he acted for six years as tutor to the young Prince Alexander of Macedon, returning to Athens when his famous pupil set out on his brief career of conquest. There he founded the famous Peripatetic School, so-called because of Aristotle's habit of walking back and forth as he taught. The doctrines which originated with the Peripatetics were destined to exert a great and lasting influence upon the development of both science and philosophy. That this influence proved to be partly bad arose from the fact that Aristotle's writings, which covered the whole field of learning, were of two sorts. His earliest studies were biological and in this area he was a scientist of erudition and originality. He observed and recorded and classified a wide range of biological forms and phenomena, and when he was thus in immediate contact with his material he wrote in the true scientific vein.

But Aristotle had in full measure that "exuberant genius for speculation" which characterized his nation. Given a few observations he was impelled to build them into a coherent philosophical system, and his specu-

¹ Not to be confused with the mathematician, Hippocrates of Chios, who was a contemporary of the physician.

lations soon outran his data. Indeed in his later writings he made statements which could easily have been put to the test of experiment, but which he never tested. Thus undeterred by inconvenient facts he outlined a system of terrestrial mechanics and fitted a small amount of data into a richly imaginative astronomical system in which he assumed that celestial motion is regulated by quite different laws from those which are operative on the earth. In thus allowing free play to his fancy Aristotle was not only disobeying his own behests, but was all unwittingly forging chains which were to bind and inhibit western thought for hundreds of years.

The Hellenistic Era

Biological science came as a relatively late development in Greece. Hippocrates lived in that Age of Pericles when classical Greek learning centered in Athens had already reached its brilliant culmination. Before his death the long Peloponnesian War had been fought to its costly conclusion. Technically it was won by Sparta, but actually all the Greek city-states including Sparta were the losers. They were left at the end (404 B.C.) torn with dissension and jealousy and weakened by twenty-seven years of intermittent fighting during which not only armies but whole communities had been destroyed. Thus the survivors fell an easy prey when in 338 B.C. Alexander's father, Philip of Macedon, struck from the north. He quickly made himself master of Greece and by the time Aristotle returned to Athens in 336 B.C. the day of the old independent city-states was past.

Although this conquest spelled the end of that first, unique expression of the Greek genius in its own homeland, it was also the beginning of a new period, known to history as the Hellenistic Age, in which the culture of Greece was preëminent throughout the ancient world. In the course of about nine years Philip's son Alexander swept triumphantly with his armies through Asia Minor, Egypt, Syria, Mesopotamia, and Persia, and even penetrated into India. Wherever he went Greek colonists followed and founded Greek towns. In this way Greek learning was carried to the people of the East, and in turn the civilizations of the East were made known to the Greeks and ultimately through them to the people of the Mediterranean world. Out of this cross-fertilization arose a new learning, predominantly Greek in expression, which was to constitute the cultural link with modern times.

Alexander died in 323 B.C. at the age of thirty-three, and his hastily forged empire fell apart. It was ultimately divided into several independent states, each governed by one of his more powerful generals. Egypt was seized by Ptolemy Lagus who established there a dynasty which lasted for three centuries. Under the Ptolemies Alexandria became the intellectual center of the Mediterranean world. The famous Museum, founded

by the second Ptolemy, developed into a kind of university where during several centuries scholars from all over the world gathered to study and teach. The Library of Alexandria ultimately housed the largest collection of books in the ancient world and numbered among its curators some of the most eminent scholars. During the five hundred years of the Alexandrian period, roughly from 300 B.C. to 200 A.D., a form of Greek was the universal language of scholars and classical Greek learning supplied the foundations on which the scholarly edifice was built.

During these years the political picture was radically changed by the emergence from obscurity of the city of Rome. It was about the time of Aristotle's birth that Rome sent out her first military expedition against a neighboring state. By 272 B.C., or roughly fifty years after Alexander's death, she had subjugated the entire Italian peninsula, including the Greek cities in the south and east. From that time until the establishment of the Roman Empire in 30 B.C. Rome went from one conquest to another until she was finally undisputed mistress of most of the known world. Then for about two hundred years the lands under Roman rule enjoyed the time of peace and consolidation and prosperity which is known as the Pax Romana. This period coincided with the last two centuries of the Alexandrian era, so that for two centuries after world government became centered in Rome Alexandria was still the acknowledged intellectual capital.

In science the Hellenistic period was most fertile in mathematics, astronomy, and physics. Euclid (ca. 330–ca. 260 B.C.), an outstanding figure in the early years, was among the first scholars to be called to Alexandria, and Archimedes (287–212 B.C.) of the Greek city of Syracuse in Sicily was probably the foremost mathematician of antiquity. Much later than these was Claudius Ptolemy,² a mathematician and geographer in whose writings was summarized the geographical and astronomical knowledge of his time. His most famous work is known as the *Almagest*. In this he outlined a cosmic system with the earth at the center surrounded by rotating planets of which the sun was one. Perhaps the most formidable task faced by scientists in the fifteenth and sixteenth centuries was the overthrow of this concept, so pleasantly flattering to the human ego.

The early Alexandrian period saw also the beginnings of physiology and anatomy as independent disciplines. Dissection of the human body was introduced and attempts were made to explain respiration and the functions of veins, arteries, and nerves. But in medicine as in cosmology the contribution which most influenced later generations was not a specific bit of original research, but a compendium of existing knowledge. Galen of Pergamum in Asia Minor (131–201 A.D.) studied at Alexandria but spent most of his active life in Rome where he acted as physician to three Emperors. For his writings he depended to some extent upon his own dissec-

² This Ptolemy was not a member of the Egyptian royal family. He wrote in the first half of the second century A.D.

tions and observations, but he also drew very largely upon the medical works of the past. In the twenty-one volumes of his which survive we have not only a summary of contemporary medical and biological knowledge but a synthesis of these into a very ingenious physical system. So satisfactory was this synthesis to Galen's successors that they made it into a medical Bible, used it as their only medical text, and finally came to regard any divergent ideas as medical heresies.

Before leaving the subject of the Alexandrian contribution to learning it may not be irrelevant to note that the later years of this period were those in which the new Christian doctrine was slowly taking form under the combined influences, especially in Alexandria, of the Mystery religions of the East, the learning of Hebrew scholars, and the teachings of the Neoplatonist philosophers who drew their inspiration from Plato and Aristotle. Thus it need not surprise us as it did the men of the Renaissance that when Aristotle's work was re-discovered a thousand years later it was found to contain much that was compatible with Christian theology.

Although the Romans had come into contact with Greek civilization when they first overran the Greek colonies in Italy, and had studied under Greek philosophers and scientists for generations, they themselves made almost no contribution to pure science. Their interests were practical and administrative, and when they did write on scientific subjects they relied for their facts on the observations of others. It is true that they drew upon contemporary medical knowledge in setting up hospitals. But the medical schools of the Roman Empire were Greek schools in which Hippocratic writings and traditions were cherished. Gradually however the spirit changed. The method of exact observation was superseded by a slavish study of the works of antiquity, and after the third century by unquestioning acceptance of the Galenic dogma. Meantime the rise of Christianity, with its emphasis on the life hereafter, also tended to discourage studies of this world. "Go not out of doors," said St. Augustine, "in the inner man dwells truth." And for several hundred years the men of western Europe obediently turned their eyes inward. These were the years after the long peace had ended, when the Western Empire was disintegrating under the pressure of barbarians on her borders and dissension within. By the beginning of the fifth century this Empire and scientific inspiration had gone into eclipse together, and the Dark Ages had begun.

Arabic Science

To understand how Greek learning was preserved and ultimately made available to western Europe, we must turn to the East. When Rome fell to the barbarians there still remained the Eastern Empire with its capital at Constantinople. This Eastern or Byzantine Empire extended from Greece to the borders of Persia and continued to maintain its independence for a further eight centuries. Here the works of antiquity were treasured

and classical learning was cultivated long after all interest in these things had failed in the West.

Thus it happened that when the heretical Nestorian Christians were driven from Constantinople in the fifth century they took with them some knowledge of Greek learning and traditions. They went first to Mesopotamia and later to Persia where they settled at Gondisapur or Jundi-Shapur. Here in the course of two centuries of scholarship they built up a rich collection of classical manuscripts and made their city a center of learning.

When in the seventh century the Arabs' surged out of the desert their first conquests brought them into contact with the Nestorians and through the Nestorian scholars with Greek learning. They were particularly impressed with Greek medicine and gradually made Greek science and philosophy the focal points around which their Moslem civilization developed. It is true that the Arabs made few original contributions to biology or medicine, but their commentaries on and translations of the classical writings conserved and spread throughout their Empire the learning of antiquity and of the later Alexandrian scholars. As their crescent of conquest spread across northern Africa and into Spain the love of Greek learning accompanied them and led to the establishment in the Iberian peninsula of some of the most famous schools of the Middle Ages. Here were accumulated treasure troves of early manuscripts in translation and of Arabic commentaries on and summaries of these manuscripts. It was largely from these Spanish centers, partly Moslem and partly Jewish, that Greco-Arabic learning first filtered into France in the knapsacks of travelers, making its way thence to Germany and England. The vitality of the Arabic-Jewish contribution to western science is attested by the survival in our own language of such Semitic words as *antimony* and *alembic*, of Persian roots in *alcohol* and *camphor* and of words derived from the Arabic of which *alizarin* and *algebra* are familiar examples.

The re-discovery of the classics which reached significant proportions in the twelfth and thirteenth centuries in western Europe had two quite different results. In the fields of art, architecture, and literature it fostered that great burst of artistic inspiration which is truly called the Renaissance. In the field of science however there followed no such freeing of the human spirit and genius. For about eight centuries scholars had been almost exclusively concerned with theology, and it was only natural that when the new learning appeared they chose to emphasize those phases of it which were compatible with their faith. Thus it was Aristotle's philosophy, which had already furnished one strand in the fabric of Christian theology, which captured their imaginations, while his scientific biological treatises were completely ignored. The *Almagest* of Ptolemy with its anthropocentric cosmology also exerted a wide influence without ever suggesting the need of corroboration. In the field of medicine, the one science

which had survived precariously through the centuries of darkness, the "vast, windy, ill-arranged treatises of Galen" had for more than a thousand years been considered a sufficient summary of medical knowledge, and the discovery of Arabian science brought no change in this point of view. Some new writings, notably those of Rhazes (865–925) and of Avicenna of Bokhara (980–1037) took their places in the medical literature, but none of them became the inspiration for a new and experimental approach.

Meantime Aristotle's doctrines were slowly acquiring a prestige which ultimately made them sacrosanct. Surely one of the saddest ironies of intellectual history is the way in which that great scientist and scholar was made the excuse in medieval times for an obscurantism and intolerance which were totally at variance with his own spirit of free inquiry. The Aristotelian dogma which proved most disastrous for chemistry was his doctrine of the four elements. According to this theory all matter is made up of the four elements, earth, air, fire, and water, in varying proportions. Obviously the word "element" as used by Aristotle has none of its modern connotations, but the years during which alchemy was gradually merging into the beginnings of chemistry were darkened and confused by attempts to attach some rational meaning to the word and to adjust the facts to fit his theory.

The Renaissance

It was not until the mid-sixteenth century that science in its modern form really began to take shape. By this time there was available a considerable body of empirical knowledge in the fields of astronomy, chemistry, physics, and medicine. Books were issuing from the new presses in ever-increasing numbers and were even beginning to carry illustrations and diagrams made from woodcuts and copper plates. Scholars were again turning seriously after a lapse of nearly two thousand years to the study of nature at first hand and to a consideration of the implications of their findings. Already the Flemish anatomist Vesalius (1514–64) was moving toward a rational approach to medicine, carrying out dissections for his students and even daring in a second edition of his book to question the dicta of Galen. Before the mid-century Copernicus (1473–1543) had completed the astronomical observations which were to shatter the Ptolemaic cosmology, and Giordano Bruno (1547–1600), that strange and wayward genius, had been born in Nola near Naples. Though not himself an observer, he brought to bear upon the data of others a clear and a questioning mind. He concluded that the universe had not been "created" but was infinite both in time and space and that the earth, far from being the center of the cosmos, moved as a planet about the central sun. For these heretical doctrines Bruno met his death by fire at the hands of the Inquisition in 1600, but the three small books which he had published continued

to spread his scientific philosophy in spite of repeated attempts to suppress them.

The intellectual revolution was given an enormous and crucial impetus by the work of another Italian whose long life was devoted entirely to experimental science. The studies of Galileo Galilei (1564–1642) in physics and astronomy, his improvement of the telescope and the compound microscope, his sponsoring of a rational interpretation of Copernicus' data and rejection of the errors of Aristotelian mechanics constitute in themselves a truly formidable contribution to science. But their influence went far beyond a revision of our ideas about the laws of physics or the structure of the universe. His experimental approach to knowledge came like a fresh breeze blowing through a closed room into a world almost suffocated by tradition and authority, and spurred men to thinking for themselves as few had thought since the days of ancient Greece. Out of this new freedom to experiment and to reason came a great upsurge in scientific inspiration which led to new discoveries in all fields, including those which were much later to be known as biochemical.

The Age of Modern Science

One of the interesting evidences of this new intellectual climate was the sudden appearance in the seventeenth century of numerous small scientific societies, often sponsored by some interested prince or noble. The Roman *Academy of Lynxes* was founded in 1603 and a Florentine *Academy of Experimentation* in 1657, the latter under the sponsorship of the Medici. In Germany the famous *Leopoldine Academy* evolved from an association of physicians which met first in 1652 at Schweinfurt to "consider medical and physical wonders and curiosities." Louis XIV supported a *French Academy of Sciences* organized in 1666, while in England a group known as the "Invisible College," which had been carrying on secret experimental studies since about 1645, was given a Royal Charter by Charles II in 1660. The *Royal Society* thus initiated is of special interest because it has an unbroken history of nearly three hundred years and is still one of the most distinguished of scientific organizations. The publication of its *Transactions* began in 1665 and except for the years 1679–1682³ has been continuous to the present.

From the beginning the Society was concerned with science in all its branches, and published side by side papers on gunnery, on blood transfusions, on mathematics and even one telling "of a Spiders not being enchanted by a Circle of Unicorn's horn, or Irish Earth, laid round about it."

³ In the early years these publications seem to have been a private financial venture of the Secretary of the Royal Society. A change in Secretaries led to suspension of publication for a few years, though published summaries of the proceedings are available. Publication of the *Transactions* was resumed when the Society guaranteed that at least 30 copies would be purchased by members!

It is of interest too that from its inception the Royal Society of London was international in its scope. Its members began immediately the voluminous official correspondence which justified the title given to the early volumes: *Philosophical Transactions giving some Accompt of the Present Undertakings, Studies and Labours of the Ingenious in many considerable parts of the World*. In 1800 the *Transactions* gave way to the *Proceedings of the Royal Society*, later divided into Series A, concerned with mathematics and physics, and Series B, which publishes research of biological interest. This last is one of the small group of journals which publish the major part of the specifically biochemical research of the present day.

By the end of the eighteenth century, science—or “natural philosophy” as it was first called—had progressed to the point where some specialization had become inevitable. Chemistry had become a separate branch of inquiry and before the end of the century Chemical Societies were beginning to be formed. At their meetings there were reported results of chemical studies of living material as well as studies of the inorganic world.

It was early in the nineteenth century that Justus von Liebig (1803–1873), having helped to lay the foundations which gave to organic chemistry an independent existence, founded in 1832 the journal which is still called *Liebig's Annalen*. Although its first title was *Annalen der Pharmacie*, since it had been formed by the fusion of two older pharmaceutical journals, it was designed to serve as an outlet for the young science of organic chemistry. This was still very close to pharmacology and medicine from which it had originally derived, and indeed it is clear that Liebig always thought of his journal as contributing to the growth of agriculture and other biological sciences. But as a matter of fact the brilliant half-century which was initiated by the synthesis of urea in 1828 saw organic chemistry moving temporarily farther and farther away from agricultural and biological interests as the new synthetic compounds proved to be better suited to the study of molecular constitution than were most of the naturally occurring organic substances. However the *Annalen der Chemie* has always carried some articles of specifically biological import side by side with those of more obviously chemical emphasis. Twentieth century biological chemistry must always be enormously indebted to the great organic chemists of the previous century, and especially to Liebig and to Emil Fischer.

It is hard to say just when and where biochemistry began to emerge as an independent discipline. It is to France that we owe the first thorough study of a substance of primary biological importance. Chevreul published in the *Annales de chimie et de physique* (founded in 1795) a series of papers entitled “Researches on Various Fatty Bodies,” and this was felt to be so important that by 1818 we find it summarized at some length in an English journal. But it was in the University of German Strasbourg that the first Department of Physiological Chemistry was

established, with Felix Hoppe-Seyler (1825–1895) as its head. In 1877 he founded the first biochemical journal, the *Zeitschrift für Physiologische Chemie*, more often referred to still as *Hoppe-Seyler's Zeitschrift* though the founder died more than half a century ago. In the introduction to the first volume Hoppe-Seyler wrote: "Die Biochemie ist hierdurch aus ihren ersten natürlichen und notwendigen analytischen Anfängen zu einer Wissenschaft erwachsen,"⁴ so that if any year is to be chosen for the birth of biochemistry perhaps 1877 is the year, since it saw the publication of the first exclusively biochemical journal. By this time scientists in America were also concerned with biochemistry and Yale had established in 1874 the first American laboratory for physiological chemistry under the directorship of Russell Chittenden.

From this point the story is too complex to be compressed into a few paragraphs. It can be read only in the journals. At the turn of the century it had become evident that the number of these must be increased to take care of the volume of research which was going forward in biochemistry. Within the space of about a year (1905–6) the *Biochemical Journal* was founded in England, the *Biochemische Zeitschrift* in Germany and the *Journal of Biological Chemistry* in the United States. With the publication of these three journals, biochemistry may be said to have come of age just twenty-eight years after Hoppe-Seyler chronicled its birth.

From the time when biochemical journals first began to be published it was obvious that they carried only a small part of the literature of interest to biochemists. The first number of the first volume of the *Zeitschrift für Physiologische Chemie* carried a list of titles of papers of biochemical importance in four other journals, three German and one French. It was only a few months later that the sixth number, the last of Volume I, listed papers from sixty-eight journals in eleven different languages, all of them having some bearing on biochemical problems. It is even more true today than it was in 1877 that "biochemical" papers may appear in any scientific journal. New analytical methods or refinements of old ones are rapidly adapted to biochemical ends. The instruments of physical chemistry and its special methods have been invaluable in the study of cell dynamics. Structural organic chemistry is obviously fundamental to any understanding of the chemical changes taking place in the cell. The task of keeping track of this vast literature is somewhat lightened by the many abstract journals in different languages, and more specifically by the indispensable *Annual Review of Biochemistry*. One volume of this publication has appeared yearly since 1932. A volume carries twenty to twenty-five articles, each written by an expert in a restricted field, and in this way there is available a reasonably up-to-date and critical summary

⁴ "Through this biochemistry has grown from its first natural and necessary analytical beginnings to a science."

of important papers published all over the world. From the very full bibliographies there given a thread can be found to lead to detailed studies of almost any problem in biochemistry.

Suggestions for Further Reading

BOYLE, ROBERT, *The Skeptical Chymist* (1661).

There is available a Modern Library edition of this book. It brings out clearly the tenacity with which men held to the Aristotelian dogmas.

DAMPIER, SIR WILLIAM C., *A History of Science*, Cambridge, 1944.

GARRISON, FIELDING H., "The Medical and Scientific Periodicals of the 17th and 18th Centuries," *Bulletin of the Institute of the History of Medicine*, July 1934.

This has been reprinted and is available as a pamphlet.

KITTO, H. D. F., *The Greeks*, Penguin, 1951.

This book, written for the Penguin Series, is a charming introduction to the story of Greek contributions to intellectual history.

NEEDHAM, JOSEPH, and PAGEL, WALTER (eds.), *Background to Modern Science*, Macmillan, New York, 1938.

The chapters of special interest in this volume are: Cornford, F. M., "Greek Natural Philosophy and Modern Science," and Dampier, Sir William C., "From Aristotle to Galileo."

ORNSTEIN, MARTHA, *The Role of Scientific Societies in the Seventeenth Century*, Chicago, 1928.

SINGER, CHARLES, *A Short History of Science*, Oxford, New York, 1941.

SPRAT, THOS., *History of the Royal Society*, first published in 1665.

Since the Society had had only a very short history when this book was written, Bishop Sprat has actually written a defense of science. The charges against which he defends it indicate the suspicion with which it was viewed.

WIGHTMAN, WILLIAM P. D., *The Growth of Scientific Ideas*, Yale, New Haven, 1951.

WOODRUFF, L. L. (ed.), *The Development of the Sciences*, Yale, New Haven, 1923.

Of special interest is Chapter VI written by the editor.

Some Selected Properties of Aqueous Solutions

The true Experimenting has this one thing inseparable from it, never to be a fixed and settled Art, and never to be limited by constant Rules.

THOS. SPRAT: *History of the Royal Society* (1665)

Many of the physical properties of water differ from those to be expected of a compound with so low a molecular weight. It is in the first place somewhat surprising that it should be a liquid at all, though this is now explained by its capacity for forming hydrogen bonds, thus giving an effective "molecular weight" much higher than that of a single molecule. Other anomalous physical properties adapt it especially well to the role it plays in living organisms. Its high specific heat prevents too rapid a rise of temperature, while its high heat of vaporization acts to lower the temperature when external conditions require it. Its unique solvent properties give it the ability to dissolve the wide range of solutes which are present in plant and animal fluids, and indeed in protoplasm itself.

Although protoplasms from different sources vary somewhat in chemical composition, they are alike in being essentially rather dilute aqueous dispersions. The inorganic ions and molecules and some of the organic compounds are present in true solution. The proteins, on the other hand, are in the colloidal state. The present chapter comprises a brief review of some of the physicochemical laws which apply to matter in these two states.

Solubility of Gases in Water

The solubility of gases in water varies with the nature of the gas, with the temperature, and with the gas pressure. Of the gases of biological interest, oxygen, nitrogen, and carbon monoxide are relatively insoluble, while carbon dioxide is considerably more soluble in water.

The solubility of gases is not usually expressed in terms of molarity or normality. In biological work the *absorption coefficient* (α) is the unit most often encountered. This is defined as the volume of a gas, *reduced to standard temperature and pressure*, which dissolves in unit volume of solvent at a specified temperature when the gas pressure is one atmosphere.

For example, the absorption coefficient of oxygen at 15°C. is 0.03415, and this means that at 15°, 1 ml. of water will dissolve an amount of oxygen which would occupy 0.03415 ml. under standard conditions. The absorption coefficients for the chief gases of biological interest are listed in Table 2-I.

TABLE 2-I. ABSORPTION COEFFICIENTS OF GASES (α)

Temperature	Oxygen	Nitrogen	Carbon Dioxide	Carbon Monoxide
0°	0.0489	0.0235	1.713	0.0354
15°	0.0342	0.0169	1.019	0.0254
25°	0.0283	0.0143	0.759	0.0214
35°	0.0244	0.0126	0.592	0.0188
40°	0.0231	0.0118	0.530	0.0178

THE EFFECT OF TEMPERATURE

A familiar example of the effect of temperature on solubility is the collection of air bubbles in a glass of tap water as it stands in a warm room. All gases are less soluble at higher temperatures and become insoluble at the temperature of boiling water.

THE EFFECT OF PRESSURE

The relationship between the weight of a gas which dissolves and the pressure of that gas was discovered by the Scots physician, William Henry

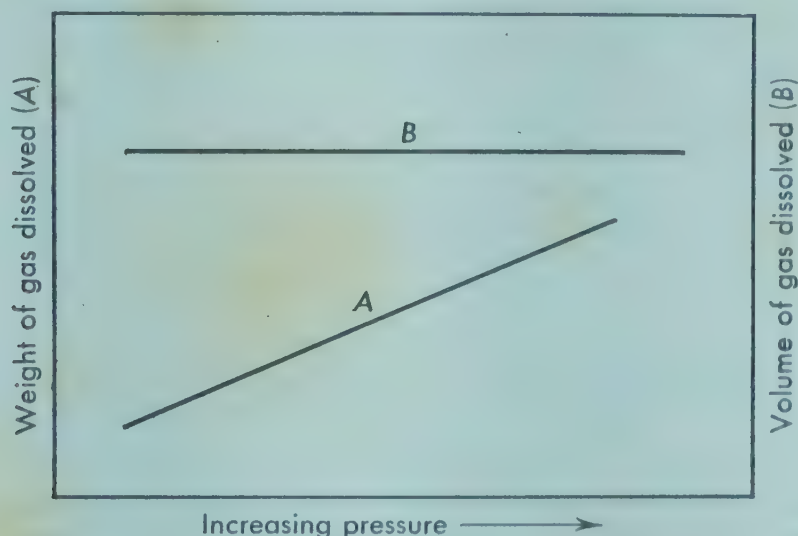


Figure 2.1. Graphic representation of Henry's Law.

(1775–1836). Henry's Law states that the weight of gas absorbed by a given volume of solvent at a fixed temperature is directly proportional to the gas pressure. Since the volume of a gas is inversely proportional to its pressure the actual volume of gas absorbed under specific experimental

conditions is independent of the pressure. The two relationships are shown graphically in Figure 2.1 in which Curve A plots the weights dissolved at various gas pressures, while Curve B shows the volume of gas, measured under experimental conditions, which dissolves at the different pressures.

When gas mixtures are in contact with a solvent the solubility of each gas is proportional to its partial pressure. Suppose for example that 15 ml. of water is to be shaken at 38°C. with a gas mixture which is 95 per cent oxygen and 5 per cent carbon dioxide. Assuming atmospheric pressure to be 760 mm., the total gas pressure will be only 760 - 49.8, or approximately 710 mm. since the gas must be saturated with water vapor. At 38° $\alpha_{O_2} = 0.024$ and $\alpha_{CO_2} = 0.55$.

$$\text{Partial pressure of } O_2 = 710 \times 0.95 = 674 \text{ mm.}$$

$$\text{Partial pressure of } CO_2 = 710 \times 0.05 = 36 \text{ mm.}$$

The 15 ml. of water will therefore dissolve

$$0.024 \times 15 \times \frac{674}{760} = 0.319 \text{ ml. } O_2 \text{ at STP.}$$

$$0.55 \times 15 \times \frac{36}{760} = 0.391 \text{ ml. } CO_2 \text{ at STP.}$$

It should be noted that Henry's Law does not hold if the gas reacts with the solvent or with some other solute. Thus for example the ability

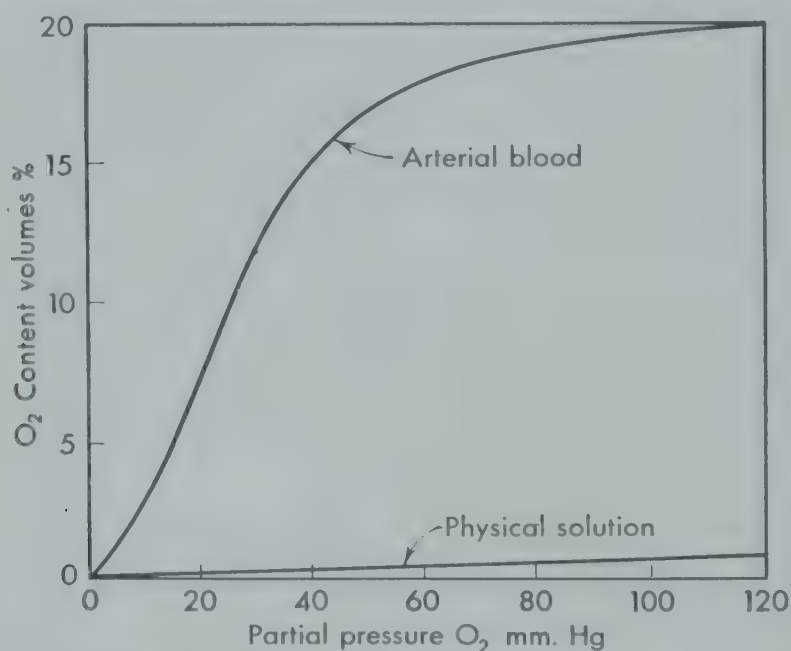


Figure 2.2. Oxygen carried in solution in plasma and in whole blood at various oxygen pressures. (From J. F. Fulton [ed.], *Textbook of Physiology*, Saunders, Philadelphia, 1949. After Barcroft).

of mammalian blood to transport oxygen depends in only very small part on its ability to dissolve the gas. By far the greater part of the oxygen present in blood is held there in loose molecular combination with the hemoglobin of the red blood cells. This is indicated graphically in Figure 2.2 which shows for increasing pressures the amounts of oxygen that are

carried in equal volumes of plasma and of whole blood with its hemoglobin. The oxygen absorbed by plasma forms a simple solution, but in whole blood there is added to the dissolved oxygen the amount of the gas which is in combination with hemoglobin at each pressure.

Use of the absorption coefficients is further limited by the fact that salts in solution lower the solubility of most gases. For example at 38°C. the absorption coefficient of carbon dioxide in water is 0.55, but in the nutrient solution which contains bicarbonate ion as well as those of sodium, calcium, potassium, and chloride, it is 0.537. In experiments on the respiration of living tissue these differences are of moment. The effect of salts upon the solubility of gases is illustrated in Table 2-II.

TABLE 2-II. INFLUENCE OF SALTS ON THE SOLUBILITY OF GASES

Gas	Solvent	Temperature (°C.)	Absorption Coefficient
Oxygen	Water	25	0.028
	0.5M NaCl	25	0.024
	1.0M NaCl	25	0.020
Carbon dioxide	Water	38	0.550
	Bicarbonate- Ringer Sol'n	38	0.537
	Serum	38	0.510

Membrane Phenomena

The living cell is a highly organized mass of protoplasm. It would be necessary to assume organization even if there were no microscopic evidence that one region of a cell differs in structure from another. The many and complex series of chemical events which are known to occur within a cell may well be only a fraction of the reactions which actually take place. But even the known processes are entirely incredible except in terms of a localization of activity and of a controlled movement of substances within the cell. At least in part, this control is achieved through the existence of membranes, some of which are microscopically visible, while others probably exist which are beyond the resolving power of our microscopes. In this sense a membrane is not to be thought of as a kind of skin made up of substances quite different from those in the fluid enclosed by the membrane. Rather a membrane results whenever at the surface of a unit or particle of solution the concentration of solute molecules is different from that in the bulk of the solution. By admitting some molecules and excluding others such membranes, enclosing various localities within a cell, control the ordered flow of solutes into those areas. Because of their differential action these membranes are spoken of as semipermeable. The

way in which the natural membranes form in living tissue is imperfectly understood, but some of the principles involved have been studied in simpler systems.

OSMOTIC PRESSURE

Several different materials have served as experimental semipermeable membranes. The earliest experiments were done with cells enclosed in their own membranes as in de Vries' work with plant cells, cited below. Red blood cells have served the same purpose and experiments on a larger scale have made use of animal bladders and of parchment paper. An artificial membrane which has been widely used consists of the gelatinous precipitate of cupric ferrocyanide deposited in the pores of an unglazed porcelain cup. Small collodion sacs are easily prepared and cellophane tubing of graded porosity is now available commercially.

When a sac of collodion or other semipermeable material is partly filled with a solution of cane sugar and then immersed in pure water, water flows into the sac. If a manometer is attached to the neck of the container it is possible to measure the pressure which develops as the solvent pushes in. Since this pressure is a result of *osmosis*, or diffusion through a membrane, it is known as the *osmotic pressure* of the sugar solution. It is a pressure which can be measured only under equilibrium conditions, that is, by allowing the solution to come to equilibrium with pure solvent through a semipermeable membrane. This pressure develops because the membrane is freely permeable to water but so retards the passage of sucrose that for experiments of short duration it may be considered impermeable to the solute. Under these conditions certain water molecules which move toward the membrane inside the sac fail to pass through because they encounter sugar molecules and are deflected by this impact away from the membrane surface. Thus water diffuses more rapidly into the sac from outside than it diffuses out from the interior where the motion of the solvent molecules is impeded by the presence of solute particles.

Two investigators are especially associated with the development of the quantitative laws governing osmotic pressure. In 1877 the German botanist Wilhelm Pfeffer (1845–1920) determined the osmotic pressures of a series of sucrose solutions, using cupric ferrocyanide in an unglazed porcelain cup as the semipermeable membrane. From Pfeffer's figures, which had shown the osmotic pressure to be proportional to the concentration of the solution and to the absolute temperature, van't Hoff was led to his classical analogy between gas pressure and osmotic pressure. That is, in the ideal case of a membrane completely impermeable to the solute the pressure for dilute solutions is given approximately by the equation

$$\pi v = nRT$$

in which π is the osmotic pressure in atmospheres, v is the volume of the solution in liters, n the number of moles of solute, R the gas constant in liter-atmospheres (0.083), and T the absolute temperature.

Osmotic Pressure in Living Cells. The first attempt to measure the osmotic pressure of living cells was made in 1888 by the Dutch botanist Hugo de Vries (1848–1935), using epidermal cells of leaves. Under normal conditions the protoplasm of these cells, within a fine plasma membrane, fills completely the space within the heavy supporting walls of cellulose, as shown in Figure 2.3, A. The cellulose shows no selective permeability; it is the plasma membrane which is semipermeable. It was found that when

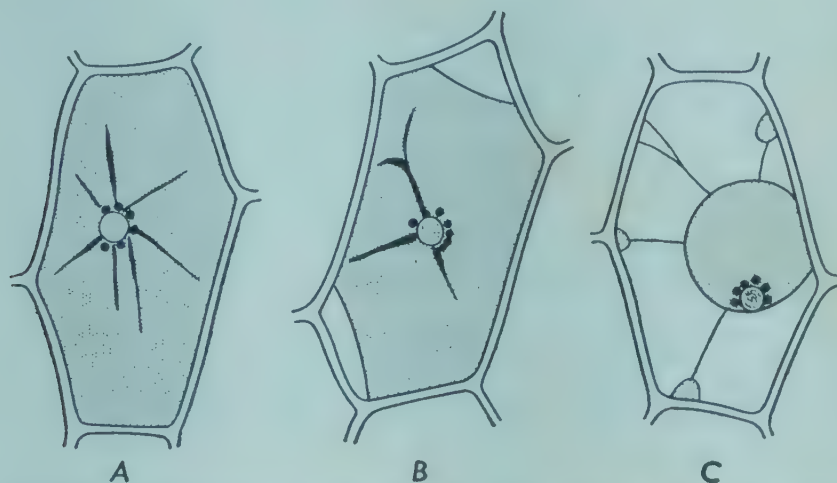


Figure 2.3. Microscopic appearance of leaf cells: A, normal cell; B, cell after immersion in 0.22M sucrose; C, cell after immersion in 1M KNO_3 . (From H. de Vries, *Z. physikal. Chem.*, 2:415, 1888.)

such cells were placed in 0.22M sucrose solutions water passed out of the cells and the plasma membrane contracted slightly as indicated in Figure 2.3, B. This escape of water from a cell is known as *plasmolysis*, and indicates that the cell osmotic pressure is less than that of the solution surrounding it. When the leaf cells were immersed in strong salt solutions they lost large amounts of water and assumed the appearance shown in Figure 2.3, C. But if the depleted cells were then transferred to pure water the plasmolysis was reversed and the cell contents again filled the space within the cellulose walls. By bathing the cells with sucrose solutions of varying concentrations, de Vries showed that water neither entered nor left the cell when it was immersed in 0.21M cane sugar solution. Such a solution is said to be *isosmotic* or *isotonic* with the cell contents, that is, to have an equal osmotic pressure. On the basis of the van't Hoff equation this pressure in the cells used by de Vries is approximately 5 atmospheres. Later determinations of the osmotic pressures of plant saps indicate that the range is wide but that for most land plants it lies between 10 and 20 atmospheres.

Experiments with animal cells similar to those of de Vries with plant tissue have given the approximate osmotic pressures of various cells and

emphasized the importance of maintaining appropriate osmotic conditions if cells are to survive. Red blood cells in pure water absorb water until their membranes are so distended that the hemoglobin escapes to give the clear red solution of *hemolyzed* blood. In order to avoid damage to the blood cells, solutions which are to be injected into the circulation are made up to be isotonic with serum. If a drug is to be administered in so dilute a solution that its own osmotic effect may be neglected, it is dissolved in isotonic or "physiological" saline, that is in a solution of sodium chloride which contains approximately 0.85 g. of the salt in 100 ml. of solution. In ordinary biochemical parlance this solution is said to be an 0.85 per cent solution.

But if the drug is to be used in appreciable concentration its own osmotic effect must be considered. Under these conditions it is necessary to determine the amount of additional solute, such as sodium chloride or glucose, which must be added to make the final solution isotonic with serum. This calculation is usually carried out by determining the amount of solute required to prepare a solution which will freeze at $-0.56^{\circ}\text{C}.$, this being the freezing point of serum. The method depends upon the fact that the effect of a solute both upon osmotic pressure and upon the freezing point is proportional to the number of particles of solute in unit volume, and hence two solutions which freeze at the same temperature will have the same osmotic pressures.

The effect of a given number of moles of solute in depressing the freezing point of a solvent depends upon whether or not the compound is ionized, and if it is, upon the number of ions which it forms. One mole of any non-electrolyte in 1000 g. of a given solvent depresses the freezing point by a fixed amount, known as the *molal depression of the freezing point* for that solvent. Thus one mole of glucose or sucrose or glycerol in 1000 g. of water forms a *molal solution* which freezes at approximately $-1.9^{\circ}\text{C}.$ In other words, the molal depression of the freezing point of water is 1.9° . But a molal solution of sodium chloride (1 mole in 1000 g. of water) shows a depression of the freezing point which is nearly twice as large, since each hypothetical sodium chloride molecule gives rise to two ions and the solution therefore contains twice as many particles as are present in an equimolal solution of a nonelectrolyte. The sodium chloride solution will also have an osmotic pressure nearly twice that of a molal glucose solution. Such a salt as zinc sulfate, on the other hand, although it also forms two ions in solution, has a smaller effect on the freezing point because the two divalent ions exert such an attraction for each other that there appear to be fewer of them. The freezing point of a molal solution of zinc sulfate is approximately $-2.1^{\circ}\text{C}.$ Salts which ionize to form three or more ions give solutions with correspondingly lower freezing points as shown in Table 2-III.

TABLE 2-III. RELATION BETWEEN FREEZING POINT LOWERING AND THE NUMBER OF PARTICLES FORMED IN SOLUTION ^a

Type of Solute	Depression of the Freezing Point of Water by 1 Mole of Solute per 1000 g. of Water (°C.)
Nonelectrolyte (glucose, sucrose)	1.9
Weak electrolyte (acetic acid, sulf- anilamide)	1.9+
One which forms 2 divalent ions (MgSO ₄)	2.1
One which forms 2 univalent ions (NaCl, HCl)	3.4
One which forms 1 divalent and 2 univalent ions (Na ₂ SO ₄ , NaHCO ₃)	4.4
One which forms 1 trivalent and 3 univalent ions (Na ₂ HPO ₄)	5.9
One which forms more than 4 ions	7.0

^a The material in this Table is adapted from a similar one in *Hospital Formulary*, University of California Press, 1941.

Suppose now that a 2 per cent solution of procaine hydrochloride is to be injected into the vein. How can it be rendered isotonic with serum? The molecular weight of the drug is 272.7 and it dissolves to form two monovalent ions.

In 100 ml. of solution there are to be 2 g. or $0.0073 \left(\frac{2}{272.7} \right)$ moles of the drug. Since this compound like sodium chloride forms two monovalent ions this will exert the same osmotic effect as 0.0073 moles of sodium chloride, or as 0.0073×58.5 g. of the salt.

$$0.0073 \times 58.5 = 0.427 \text{ g.}$$

Since 0.85 g. of sodium chloride in 100 ml. of solution is isotonic with serum, there must be added to 100 ml. of the procaine hydrochloride solution, $0.85 - 0.43 = 0.42$ g. of sodium chloride.

If glucose were to be used instead of sodium chloride it would be necessary to take account of the fact that it is a nonelectrolyte. Thus 0.0072 moles (0.42 g.) of sodium chloride is equivalent to nearly 0.0144 moles of unionized glucose in osmotic effect, and approximately 2.6 g. of glucose (0.0144×180) added to 100 ml. of 2 per cent procaine hydrochloride would give a solution of the required osmotic pressure.¹

Permeability of Living Membranes. Undoubtedly tissues and organisms differ greatly in the permeability of their membranes. Furthermore many

¹ In hospitals where this sort of calculation must be made frequently, Tables are available showing the sodium chloride equivalents of the commonly used drugs. See for example the *Hospital Formulary* issued by the University of California and published by the University of California Press, 2nd ed., 1952.

of the lower forms such as molds and bacteria possess great adaptability in this matter and can be trained over a period of time to survive in media which are far from the norm. It is therefore possible to speak of the permeability of living membranes only in most general terms.

The one substance to which all living membranes seem to be completely permeable is water. When we consider other small molecules and ions the evidence is conflicting. Many animal and plant cells lose water when placed in concentrated aqueous solutions of salts or of various soluble organic compounds. This would indicate that their membranes are impermeable to these solutes. Yet clearly these very substances must find their way into living cells, for it is only in this way that cells can obtain food or maintain the ionic ratios which are essential to them. Perhaps the experimental effects observed are a result of a difference in the rates at which water and the water-soluble substances penetrate the membrane. A slow attainment of equilibrium on the part of the solutes would give rise to a preliminary plasmolysis which would reverse as equilibrium was attained.

In contrast to their retarded permeability for water-soluble molecules, cell membranes are much more readily permeable to substances which resemble the fats in solubility. This fact has led to the theory that the cell membranes consist at least in part of fatty material and that substances penetrate a membrane by dissolving in the material of which it is made.

SURFACE EQUILIBRIA

Some indication of the way in which cell membranes form may be obtained from a consideration of the energy relations which obtain at a surface.

Surface Tension. When a drop of liquid falls freely it assumes a spherical form because of unbalanced forces of molecular attraction. A molecule in the center of the drop is attracted in all directions by surrounding molecules. But while molecules on the periphery are attracted inward, this attraction is not balanced by any comparable attraction between the surface molecules and the air. As a result there is at the surface an amount of free surface energy which depends in part on the surface area and for the rest on what is known as the *surface tension* of the liquid. This tension is a measure of the work required to form unit area of additional surface, and is expressed in units of force and length, usually dynes per centimeter. The actual free energy at a surface, F_s , is then expressed

$$F_s = \gamma A$$

in which A denotes the area of the surface and γ the surface tension. Thus when a drop assumes spherical form it is reducing as far as possible the

free surface energy, since a sphere presents the smallest possible surface area for a given volume.

The free energy of any system tends toward a minimum at constant temperature and pressure. As just indicated, a freely falling pure liquid exemplifies this tendency when it assumes spherical form. When instead of a pure liquid a solution is allowed to fall freely there is available a second resource for reducing the free surface energy. In general the surface tension of a solution differs from that of the pure solvent, being greater or less, depending upon the nature of the solute and upon its concentration. Many organic compounds in solution cause a significant lowering of surface tension, and these substances are said to be *surface active*. Such solutes, by collecting in greater concentration at the surface than in the bulk of a solution, reduce the surface tension and therefore the free energy of the system. Such a concentration of excess solute molecules in a thin layer at a surface is one aspect of the phenomenon known as *adsorption*. Some solutes, among them strong electrolytes, cause slight increases in surface tension. These *surface inactive* compounds tend to migrate into the center of a drop and away from the surface, thus lowering the surface tension.

When a liquid surface is in contact with that of another liquid the boundary between the two is known as an *interface*. In general, as shown in Tables 2-IV and 2-V, interfacial energies are less than those which obtain at a liquid-gas interface. When one or both of the liquids is a solution, surface active solutes collect at the interface as they do at other surfaces, thus reducing the interfacial tension.

Measurement of interfacial tensions between living cells and their environment has revealed very low values, as for example 1.3 dynes for frog leukocytes suspended in frog plasma. This points clearly to a high con-

TABLE 2-IV. SURFACE TENSION OF LIQUIDS ^a

Liquid	Concentration	Temperature (°C.)	Surface Tension (dynes/cm.)
H ₂ O		20	72.75
H ₂ O		25	71.97
Acetone		20	23.7
Chloroform		20	27.1
Ethyl alcohol		20	22.3
Ethyl acetate		20	23.9
Ethyl ether		20	17.0
aq. NaCl	0.1M	20	72.92
aq. CaCl ₂	0.1M	25	72.32
aq. MgCl ₂	0.1M	20	73.07
aq. ethyl alcohol	5.21 per cent	25	54.87
aq. acetic acid	5.0 per cent	25	55.45

^a Measurements were taken with the liquids in contact with air.

TABLE 2-V. INTERFACIAL TENSION BETWEEN TWO LIQUIDS

Liquids	Tempera- ture (°C.)	Surface Tension (dynes/cm.)
Water/olive oil	23	17.0
Water/isoamyl alcohol	23	4.4
Water/benzene	20	35.0
9% Ox gall/olive oil		7.2
Soap sol'n/olive oil		3.65
Water/ethyl ether	23	10.9
Water/oleic acid	25	12.8

centration of surface active material at the boundary, and has led to the suggestion that the cytoplasmic membrane is simply a heightened concentration of certain cell constituents at the surface of the cell. Among the compounds universally present in living cells are certain complex fatty substances known as phospholipids which depress the surface tension markedly. A concentration of these compounds at the cell surface would account for the permeability of the cell membrane to fat-soluble materials, and would also satisfy that tendency of all systems to reduce their free surface energy.

Adsorption on Solid Surfaces. The removal of toxic gases from air by finely divided charcoal is a familiar procedure, as is decolorization of solutions by certain forms of the same substance. These are both examples of adsorption, a substance in the gas or liquid phase forming a concentrated film on the solid surface. It has been shown that the amount of material adsorbed is proportional to the area of the interface, but depends also on the concentration of the material which is to be adsorbed and on the solvent employed. In general a substance is most completely adsorbed from the solvent in which it is least soluble. We shall see later how this process of adsorption on solid surfaces has been adapted to the separation of complex mixtures.

Light Absorption

A substance which absorbs some part of the visible spectrum transmits light of the complementary wave lengths and appears colored. Substances which absorb light of longer or shorter wave length than the visible ones appear colorless. Both types of absorption have found wide use in biochemistry.

THE SPECTRUM

Light consists of a corpuscular form of energy, the quantum, which also exhibits the characteristics of waves. In Figure 2.4 the undulating line represents an advancing wave front. The distance from *A* to *B*, from one

wave crest to the next, is the *wave length*. The number of complete waves in some unit length, as for example in the distance between *C* and *D*, is the *wave number*, while the *frequency* indicates the number of wave crests which pass a point in space in some unit time. Using λ to stand for wave

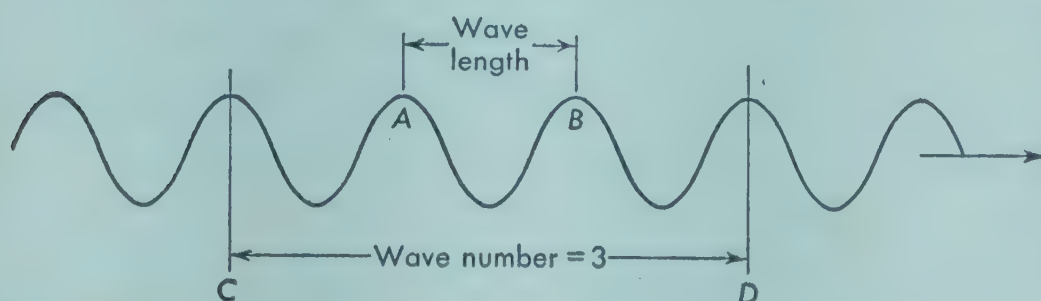


Figure 2.4. Relation between wave length and wave number.

length, σ for wave number, ν for frequency and c for the speed of light (3×10^{10} cm. sec.⁻¹), the relations between these quantities may be expressed

$$\frac{1}{\lambda} = \sigma = \frac{\nu}{c}$$

The wave lengths of various vibrating forms of energy range from approximately 10^8 meters for ultrasonic waves to 10^{-12} meters for the

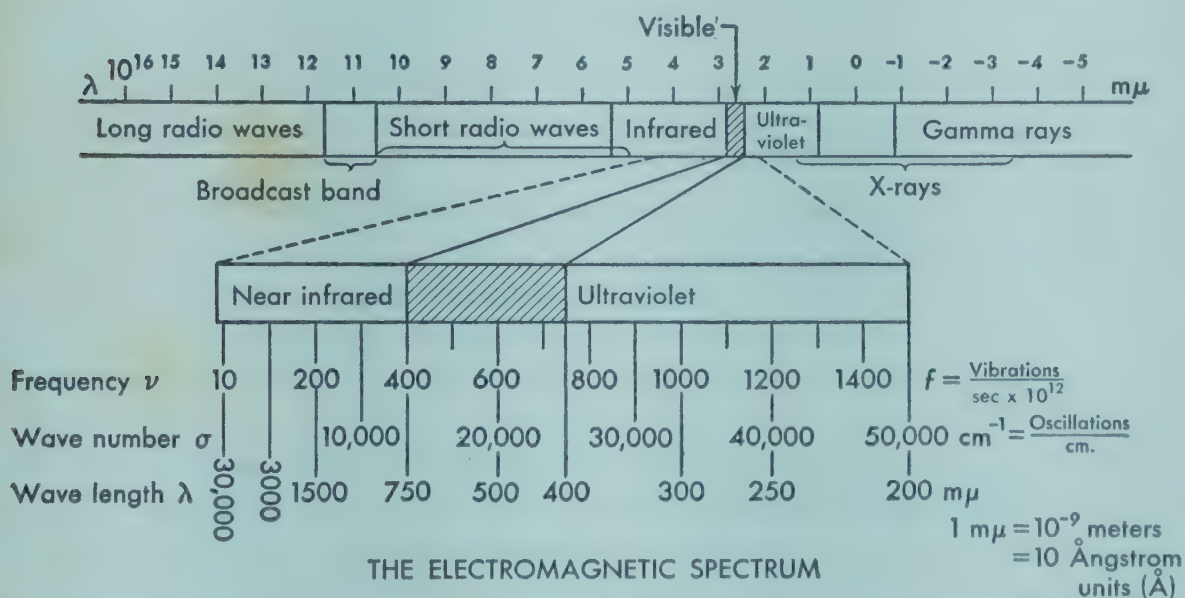


Figure 2.5. The spectral distribution of radiant energy with the units in which it is measured.

gamma radiation from radioactive elements. The wave lengths of cosmic rays are still shorter than this. Figure 2.5 shows the electromagnetic spectrum, in which the narrow shaded band marks the portion which produces the sensation of visible light. In the lower part of the figure is represented the spectral region which is commonly used for spectroscopic

measurements, with the three descriptive terms indicated in the appropriate units.

The wave length of light is measured in different units, depending on the part of the spectrum which is involved. The longer waves are measured in meters. For wave lengths from those of the ultraviolet through the visible and the infrared the unit is sometimes the millimicron ($m\mu$) which is 10^{-7} cm., and sometimes the Ångstrom unit, named for the Swedish physicist A. J. Ångstrom (1814-1874). This unit is abbreviated Å or AU and is one tenth of a millimicron or 10^{-8} cm. The conversion of wave lengths to wave numbers and frequencies must take account of the units in which the desired values are to be expressed.

The wave number is usually reported as the number of waves per centimeter (cm.^{-1}). If a wave length of 400 $m\mu$ (4000 Å) is to be converted to wave number it must first be expressed in centimeters.

$$400 \times 10^{-7} = \lambda \text{ in cm.}$$

$$\frac{1}{400 \times 10^{-7}} = \frac{1}{400} \times 10^7 = 25,000 \text{ cm.}^{-1} = \sigma$$

The frequency is occasionally expressed in waves per second, but since these numbers are very large the more usual unit is the fresnel (f) which gives the number of vibrations per 10^{-12} seconds.

$$\sigma = \frac{\nu}{c}$$

$$25,000 = \frac{\nu}{3 \times 10^{10}}$$

$$\nu = 25,000 \times 3 \times 10^{10} = 7.5 \times 10^{14} \text{ vibrations/sec.}$$

$$\nu = \frac{25,000 \times 3 \times 10^{10}}{10^{12}} = 75,000 \times 10^{-2} = 750 \text{ f.}$$

COLORIMETRY

Many compounds of biochemical importance can be converted into derivatives which are so brightly colored that very small amounts of them can be estimated quantitatively by taking advantage of their absorption of light in the visible range. Quantitative colorimetric methods depend upon relationships summarized in the Lambert-Beer Law.

Lambert studied the relation between the original intensity of a parallel beam of monochromatic light and its intensity after passing through various *thicknesses* of a colored solution. He found that the intensity is reduced by some fractional amount in passing through equal thicknesses of the absorbing solution. Thus if it is reduced by half in the first centimeter it will be reduced by half again in traversing the next centimeter,

and so on. Allowing I_0 to stand for the intensity of the original or incident light, I for the intensity of the transmitted light and d for the thickness of the layer of solution traversed, Lambert's results may be expressed

$$\log \frac{I_0}{I} \propto d$$

or inserting the proportionality constant

$$\log \frac{I_0}{I} = k_1 d$$

The k_1 is known as the *extinction coefficient*, the value of which depends upon the concentration of the solution, the wave length of the light, and the temperature.

Beer investigated the relation between the *concentration* of a colored solution and the amount of monochromatic light which it absorbed. He found that under suitable conditions the absorption is proportional to the number of solute molecules in unit volume. This may be expressed

$$\log \frac{I_0}{I} \propto c \text{ (concentration)} \quad \text{or} \quad \log \frac{I_0}{I} = k_2 c$$

in which k_2 is a constant similar to k_1 above. By combining the two equations the relation between concentration, solution thickness, and absorption is given by the single expression

$$\log \frac{I_0}{I} = Kcd$$

If the concentration is expressed in moles per liter and the thickness, d , in centimeters, K is known as the *molar extinction coefficient*, usually designated ϵ .

Colorimetric methods all involve a direct or indirect comparison of the absorption of light by a standard solution with its absorption by a solution containing the same colored solute in unknown concentration. In practice this is often done by varying the thickness of the solution of the unknown until it absorbs the same fraction of the incident light as is absorbed by a known thickness of the standard solution. Under these circumstances $\log \frac{I_0}{I}$ becomes the same for the two solutions. Therefore

$$Kc_1d_1 = Kc_2d_2$$

or

$$\frac{c_1}{c_2} = \frac{d_2}{d_1}$$

Since the two thicknesses are measured by the instrument and c_1 or the concentration of the standard is known, c_2 can be calculated.

There are a number of different instruments available for colorimetric work. In the simplest type, ordinary white light is used and the two solutions are compared directly. In others a narrow spectral band, isolated by means of filters, is sent through the colored solution and the intensity of the transmitted light is measured with a photoelectric cell. For a discussion of these instruments with their advantages and disadvantages a laboratory manual should be consulted.

ABSORPTION SPECTRA

There are two types of spectra which are used in the investigation of atoms and molecules. These are *emission* and *absorption* spectra. When a substance is exposed to some source of energy such as an electric spark or a flame, its electrons are pushed to outer or higher energy levels. As they fall back to lower levels the energy which they have just acquired is released as radiant energy. If this emitted light is analyzed by a spectroscope there is obtained an emission spectrum consisting of a set of bright lines or bands against a dark background.

An absorption spectrum is the opposite of an emission spectrum. When radiation, visible or invisible, passes through an absorbing medium some wave lengths are absorbed and others are transmitted. Thus the spectrum obtained when the transmitted light is analyzed consists of a set of dark lines or bands, representing the absorbed light, against a bright background made up of the transmitted wave lengths. Whereas emission spectra are of special importance in the identification of elements, each of which gives out a highly characteristic set of radiations when suitably excited, absorption spectra are widely used in the study of molecules, especially those of organic compounds. These spectra may be obtained not only with visible light but with radiations in both the ultraviolet and the infrared ranges.

Theory of Absorption. It was recognized by the early dye chemists that color is associated with the presence of certain groups in an organic molecule. These are known as chromophore groups, nearly all of which prove in practice to be unsaturated in some degree. According to modern theory all organic molecules absorb light energy, but the only compounds which absorb in the visible range are those which are capable of resonance, that is, of having more than one stable electronic configuration.

When energy is absorbed by a compound it may cause three different kinds of change in the molecule, the total energy change in any given case being the sum of the three. Electrons may be pushed to higher energy levels; the extent of vibration of atoms within the molecule may change and finally the rotation of the molecule as a whole about its center of mass may be altered. In order to induce one of these changes in a specific organic grouping the radiant energy provided must include the specific wave lengths which that group can absorb. Other wave lengths which

may be present but are not absorbed by the compound in question, are transmitted. In general, changes in the vibrational and rotational states involve small energy changes and these are usually measured by infrared absorption spectra. Changes in the electronic state of a compound on the other hand involve much larger amounts of energy and are induced by absorption of light of shorter wave length or higher frequency, usually in the ultraviolet but occasionally in the visible region.

Measurement of Absorption. The simplest possible instrument for determining the absorption of visible light is the spectroscope. In such an instrument the entering white light is spread into a spectrum by means of a prism. When some colored substance is put in the path of the light beam, the absorbed wave lengths are indicated by dark bands on the bright solar spectrum.

For more exact work it is necessary to use a spectrophotometer, an instrument which measures light absorption electrically and in which the wave length of the absorbed light can be determined precisely. In very general and greatly simplified terms a spectrophotometer works as follows. The incident beam of radiant energy is broken into a spectrum by a grating or prism. From this spectrum narrow bands, as nearly homogeneous as possible, are isolated and passed one at a time in sequence through the solution which is being examined. The intensity of the transmitted light is measured for each wave length by means of a photoelectric cell.

Spectral data are reported in the form of curves in which either the wave length or wave number (cm.^{-1}) or frequency (f) is plotted on the abscissa and some value which indicates the extent of absorption is used as ordinate. It is customary to give the absorption in terms of one of the extinction values. For example the *specific extinction coefficient* (K) can be calculated from the expression $\log \frac{I_0}{I} = Kcd$ if c is the concentration in grams per liter and d the thickness in centimeters. Or the *extinction coefficient* (E) may be used, this being equal to $\log \frac{I_0}{I}$. The third common extinction unit is the *molecular extinction coefficient* (ϵ) which is equal to the specific extinction coefficient multiplied by the molecular weight of the compound. The use of any one of these values as ordinate gives a curve in which a peak indicates a high absorption of that particular wave length. Thus when the absorption is high, " I ," which measures the intensity of the transmitted light, is low, making the $\log \frac{I_0}{I}$ correspondingly high. In some graphs the ordinate unit is the opposite of an extinction unit, and the effect of each wave length is then recorded in terms of its transmittancy (T), or the actual percentage of the incident light which is transmitted. On such a graph high absorption values correspond to low transmittancies. In Fig-

ures 2.6 and 2.7 are shown two absorption curves in which extinction values are used, while the infrared absorption curves of Figure 2.8 are plotted in terms of transmittancy.

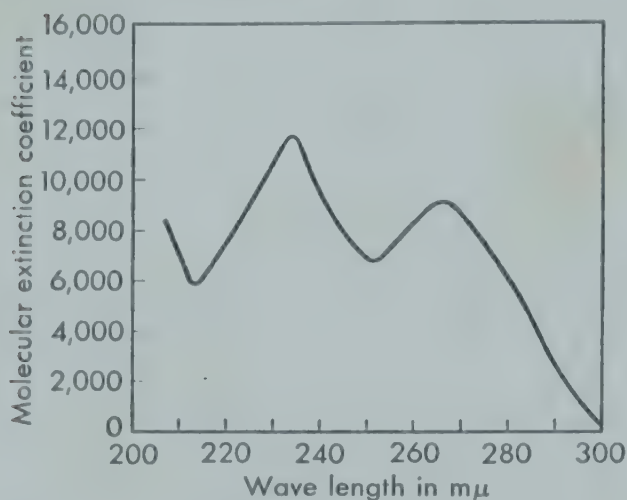


Figure 2.6. Ultraviolet absorption of vitamin B₁ in water solution. (From O. Wintersteiner, R. R. Williams, and A. E. Ruehle, *J.A.C.S.*, 52:519, 1935.)

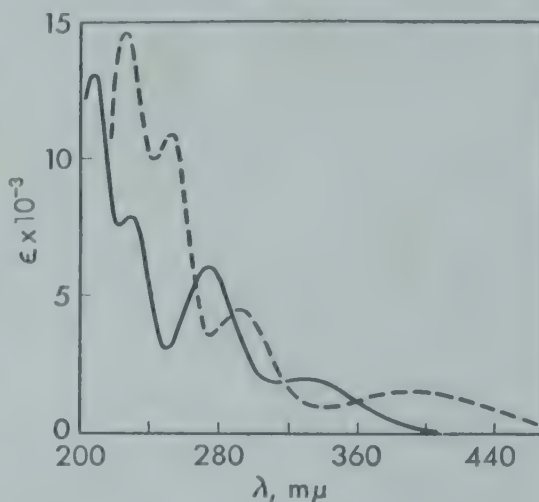


Figure 2.7. Ultraviolet absorption of *m*-nitrophenol: solid line in pH 3; broken line in 0.1N NaOH. (From L. Doub and J. M. Vandenbelt, *J.A.C.S.*, 71:2414, 1949.)

Infrared Absorption. Infrared absorption, except that it involves radiant energy of low frequencies, is like absorption in the other spectral regions. But an infrared spectrum can be interpreted more specifically than those of the visible or ultraviolet ranges. It was noted earlier that the changes induced in a molecule when it absorbs infrared radiation are changes in molecular rotation or in atomic vibration. Actually most of the instruments now in use generate radiations in the near infrared ($3\ \mu$ to $25\ \mu$) and these frequencies affect only the vibrational states. It has been found that while part of the absorption in the near infrared is referable to the molecular structure as a whole, other frequencies absorbed are charac-

TABLE 2-VI. WAVE NUMBERS OF INFRARED ABSORPTION BANDS DUE TO SPECIFIC ORGANIC LINKAGES ^a

Group	Wave Number (cm. ⁻¹)
C—C	1000
C—Cl	750
C=C	1640
C=C (conjugated)	1590
C=C (aromatic)	1595
C=O (ester)	1745
C=O (acid)	1710
C=O (ketone)	1695

^a Figures from G. R. Harrison, R. C. Lord, and J. R. Loofbourov, *Practical Spectroscopy*, Prentice-Hall, New York, 1948.

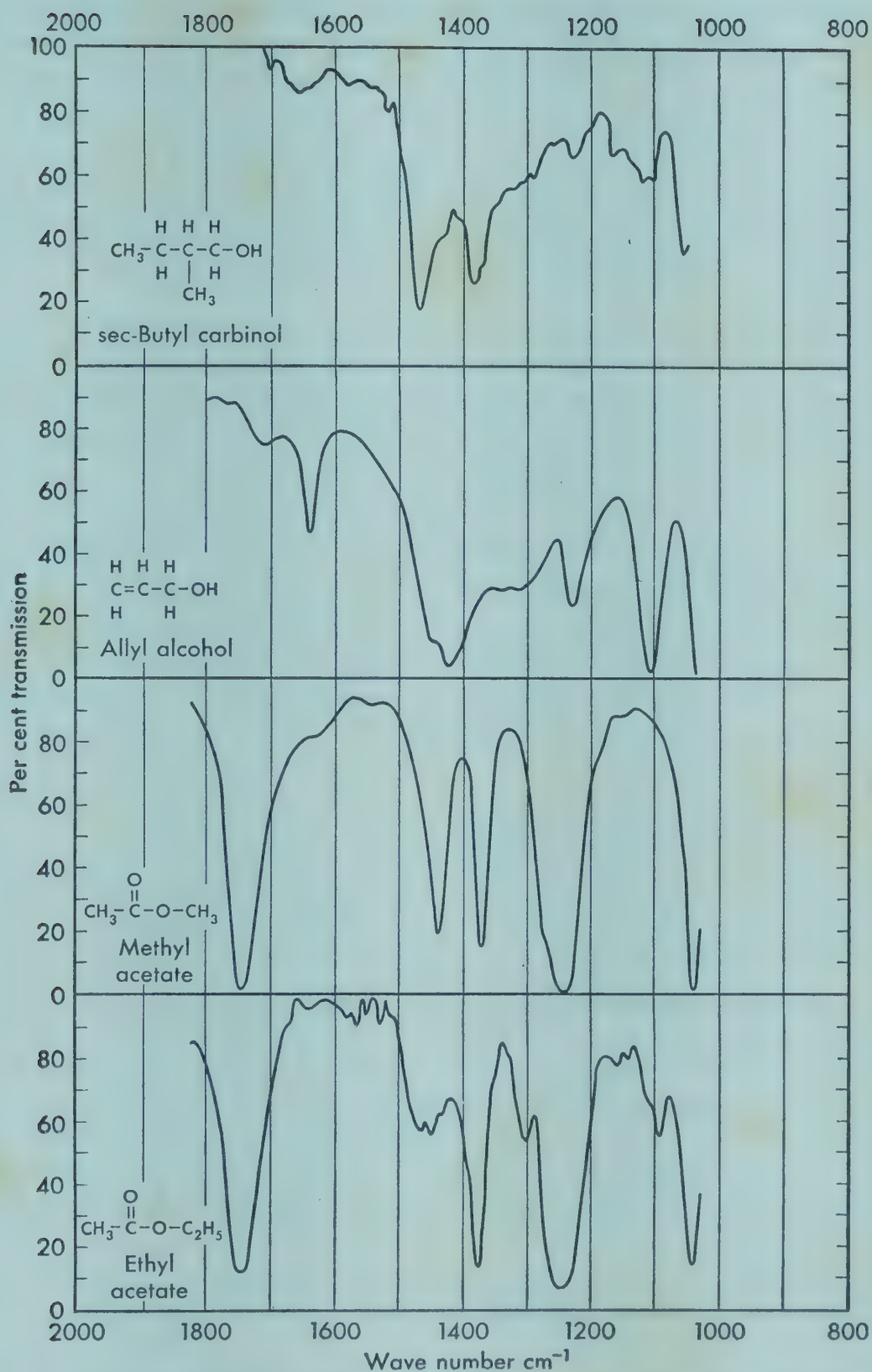


Figure 2.8. Infrared absorption curves for four simple organic compounds. Note the absorption at 1640 cm^{-1} in the allyl alcohol curve, referable to the double bond, and those at 1745 in the ester curves, referable to the ester carbonyl group. (From R. B. Barnes, U. Liddel, and V. Z. Williams, *Ind. and Eng. Chem., Anal. Ed.*, 15:659, 1943. By permission of the American Chemical Society, copyright owner.)

teristic of small groups within the molecule. Whenever a given group is present, absorption of its particular frequency appears in the molecular spectrum, which is thus a summation of the bands due to the groups and those due to the gross molecular structure. Table 2-VI lists the wave numbers of the energy absorbed by a few of the common organic groupings. It should be noted that even the slight differences between carbonyl groups in ester, acidic, and ketonic linkages give rise to absorptions of different wave lengths. Because of this specificity the infrared spectrum of a compound has been called its "finger print."

For reasons which need not be elaborated here, infrared absorption curves are usually reported with transmittancy rather than extinction values plotted against wave length or wave number. In such curves, of which Figure 2.8 is an example, the largest absorption is indicated by those portions of the curve which are nearest the base line.

Organic chemical analysis by means of infrared absorption is becoming increasingly important. This is particularly true in the biochemical field since many important compounds are colorless and also absorb very little in the ultraviolet. Infrared spectra of such substances not only identify specific groups but give an indication of the structure of the molecule as a whole. In dealing with substances as complex as are many of those elaborated by living tissue, infrared spectroscopy is a tool of extraordinary convenience and precision.

Hydrogen Ion Concentration

The acidity of a living cell varies within very narrow limits, and work with biological systems is possible only in media in which a specified acidity can be maintained. For example, the cell catalysts or enzymes are extremely sensitive to variations in acidity, and may be completely inactivated by slight increases or decreases in the hydrogen ion concentration. Similarly the acidity of the soil determines the type of plant which can be grown on it, and optimum growth of bacteria is possible only if the medium is adjusted to maintain the correct acidity in the face of metabolic fluctuations in the actual acid content. Thus the measurement and control of hydrogen ion concentration are of fundamental importance in all types of biological work.

The units to be used in any quantitative discussion of ionic equilibria depend upon the concentrations of the solutions to be considered and upon the possible accuracy of the experimental procedures. The quantitative laws were originally worked out in terms of ion concentrations but it has long been known that, because of interionic attractions, the operative factor is not the actual concentration but something which has been called the "effective ion concentration" or the "activity." The activity of the ions in a solution of a specific concentration may be determined experimentally. The ratio of this activity to the actual concentration of a

particular ion gives the "activity coefficient" of that ion, a quantity which is usually represented by the letter f .

$$\frac{a}{c} = f$$

When the activity coefficient is known the activity of an ion can be calculated as the product of its concentration by the coefficient.

$$a = fc$$

The activity coefficient is not constant for any one ion, but varies both with its own concentration and with the concentrations of other ions present in the solution. In very accurate formulations this factor must always be taken into account. But as it happens the solutions commonly used in biochemical work are relatively dilute, and at that concentration level the difference between concentration and activity is so small that it may usually be neglected. The following discussion is therefore couched in the simpler terms of concentrations, but it must be borne in mind that these terms are applicable to only a limited concentration range and that the results obtained are approximations.

THE pH SYSTEM

Although the range of hydrogen ion concentrations with which biochemists are concerned is relatively small, it may well include several hundred or even several thousand molar units. The difficulty in choosing a scale for a graph in which the hydrogen ion concentrations may range, for example, from 10^{-5} to 10^{-8} moles per liter, has led to the widespread adoption of Sørensen's ² pH system. The p stood originally for the French *puissance* but has been generally adopted since it serves equally well for the German *Potenz* and the English "power" used in its mathematical sense. In this system then the acidity is expressed as a power of the hydrogen ion concentration.

The pH is defined most simply as the *negative logarithm* of the hydrogen ion concentration. With values which are simple powers of ten the transformation is simple. The negative logarithm of a number is the logarithm of its reciprocal. Hence the pH corresponding to a hydrogen ion concentration of 10^{-3} is 3.

$$\text{pH} = \log \frac{1}{10^{-3}} = \log 10^3 = 3.00$$

² Søren P. L. Sørensen (1868–1939) was born in Sweden but spent the greater part of his life in Copenhagen. There in 1901 he succeeded Johann Kjeldahl as Director of the Chemistry Department of the Carlsberg Laboratory, and much of his work appeared in the *Reports* of that Laboratory. He is best known for research on the physical chemistry of the amino acids and the proteins.

For a hydrogen ion concentration of 1.3×10^{-3} the pH is obtained as follows:

$$pH = \log \frac{1}{1.3 \times 10^{-3}} = \log \frac{10^3}{1.3} = \log 10^3 - \log 1.3 = 3.00 - 0.11 = 2.89$$

By the use of the pH system the range of acidities from that of normal hydrochloric acid to that of normal sodium hydroxide may be expressed in 15 units from zero to 14, as shown in Table 2-VII. This gives a con-

TABLE 2-VII. HYDROGEN ION CONCENTRATIONS AND pH VALUES AT 25°C.

Hydrogen Ion Concentration	Hydroxyl Ion Concentration	Solution	pH
10^0	10^{-14}	1N HCl	0
10^{-1}	10^{-13}	0.1N HCl	1
10^{-2}	10^{-12}	0.01N HCl	2
10^{-3}	10^{-11}	0.001N HCl	3
10^{-12}	10^{-2}	0.01N NaOH	12
10^{-13}	10^{-1}	0.1N NaOH	13
10^{-14}	10^0	1N NaOH	14

venient scale for plotting experimental results. For example, an acidity change from 10^{-5} to 10^{-7} would require 100 units, but in terms of pH needs only 2.

To convert pH values back to hydrogen ion concentrations the process outlined above is reversed. Suppose that the pH is 5.9. Either of the two following procedures will give the corresponding hydrogen ion concentration.³

$$1. \quad 5.9 = \log \frac{1}{[H^+]}$$

$$\text{antilog } 5.9 = 800,000 = \frac{1}{[H^+]}$$

$$[H^+] = \frac{1}{8 \times 10^5} = \frac{1}{8} \times 10^{-5} = 0.125 \times 10^{-5} = 1.25 \times 10^{-6}$$

2. Since 5.9 is the negative logarithm, the logarithm is -5.9 , the *entire number* being negative. But in logarithm tables the mantissa is always given as a positive number, hence this value must be rewritten with a **negative characteristic and a positive mantissa**.

³ In the equations that follow, square brackets indicate as usual the concentration in moles per liter.

$$-5.9 = -6 + 0.10 = \bar{6}.10$$

$$\text{antilog } -6 = 10^{-6}$$

$$\text{antilog } 0.10 = 1.25$$

$$[\text{H}^+] = 1.25 \times 10^{-6}$$

WEAK ELECTROLYTES

Since the chief weak electrolytes of biochemical interest are weak acids, the discussion which follows is in terms of these compounds and their salts. Obviously the principles deduced are applicable also to weak bases and the salts which they form.

Weak acids exist in solution in equilibrium with small concentrations of their ions. The ionization of such an acid, designated HA, is most simply indicated by the equation



it being understood that the ions are more or less hydrated. Application of the Mass Action Law to this equilibrium gives

$$\frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]} = K_i$$

K_i is the ionization or dissociation constant and is a measure of the strength of the acid, being larger for more highly dissociated acids and numerically very small for the weaker ones. Sometimes this constant is designated K_c , meaning that it is obtained by substitution of concentration values in the equilibrium expression. In contrast K_a is the more useful constant obtained by the use of activities in place of concentrations.

$$K_a = \frac{a_{\text{H}^+} \times a_{\text{A}^-}}{a_{\text{HA}}} = \frac{f[\text{H}^+] \times f[\text{A}^-]}{f[\text{HA}]}$$

Titration Curves. *Monobasic Acids:* In Figure 2.9 are shown the curves obtained when two different tenth molar acids are neutralized with tenth molar base. For comparison there are given also the pH values at which some of the common indicators change color. The curve for hydrochloric acid is typical of those given by strong acids. Being completely ionized in solution, its hydrogen ion concentration is nearly 10^{-1} or its pH is nearly unity before any base is added. When the acid is 99 per cent neutralized the pH has increased only to about 3, but the drop of alkali which completes the neutralization changes the pH value suddenly from slightly more than 3 to 9 or 10. Thus for this neutralization any of the common titration indicators could be used, though neutral red which changes color at the point of inflection of the curve would be most suitable.

The curve is different with the weak acetic acid. Its original pH is higher (2.75) and as soon as base is added the resulting salt suppresses the ionization of the remaining acid and raises the pH sharply. Thereafter the pH rises gradually while about three quarters of the base is added, and again more sharply as the equivalence point is reached. The point

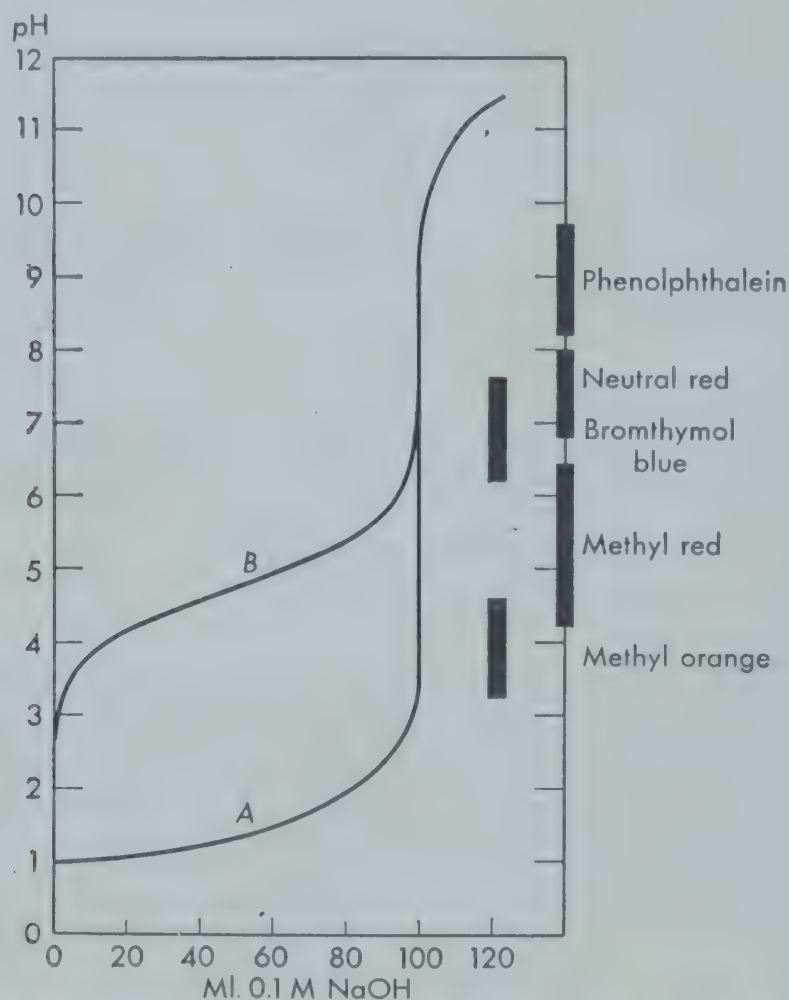


Figure 2.9. Titration of 100 ml. 0.1M hydrochloric (curve A) and of 100 ml. 0.1M acetic acid (curve B) with 0.1M sodium hydroxide.

of inflection of the curve for acetic acid comes at about pH 8.7, which is close to the pH at which phenolphthalein and cresol red change color. Either of these indicators may therefore be used to mark the end point in titration of acetic acid with a strong base.

Polybasic Acids: Polybasic acids ionize in steps, giving ionization constants of which the primary is always larger than the secondary, and the secondary larger than a tertiary.

Figure 2.10 shows the curve obtained when 0.1M phosphoric acid is titrated with 0.1M sodium hydroxide. The point of inflection at about pH 4.4 results from the neutralization of the first hydrogen. This end point can be determined by the use of methyl orange as indicator. At

the second equivalence point, when the second hydrogen has been neutralized, the pH is about 9.6 so that titration to a phenolphthalein end point marks the neutralization of the first two hydrogens. Table 2-VIII gives the activity constants for a few common weak acids and bases. Since the constants themselves involve negative exponents a value designated as pK_a is frequently used in their stead. The pK_a is the negative logarithm of the activity constant.

BUFFERS

The long gradual slope in the titration curve for a weak acid indicates that the pH of a mixture of a weak acid and its salt changes only slightly as the ratio of the constituents varies. For example, a mixture of acetic acid and sodium acetate contains acetate ion, undissociated acetic acid, and a small amount of hydrogen ion. Addition of more hydrogen ion brings about the formation of additional undissociated acid, since there is a large reserve of acetate ion

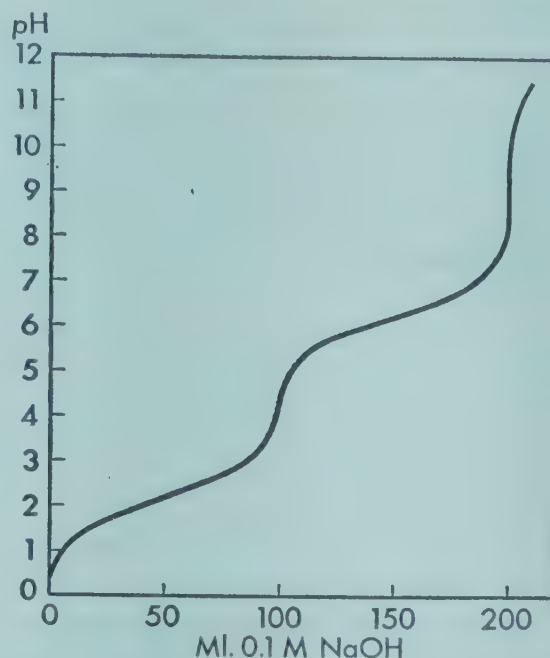


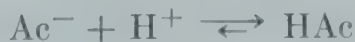
Figure 2.10. Titration of 100 ml. of 0.1M orthophosphoric acid with 0.1M sodium hydroxide.

TABLE 2-VIII. ACTIVITY CONSTANTS (K_a) OF WEAK ACIDS AND WEAK BASES AT 25°C.^a

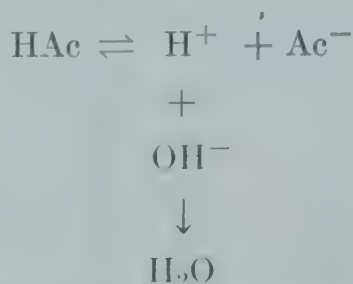
Acid	Primary Dissociation		Secondary Dissociation		Tertiary Dissociation	
	K_a	pK_a	K_a	pK_a	K_a	pK_a
Acetic	1.75×10^{-5}	4.76				
Benzoic	6.29×10^{-5}	4.20				
Carbonic	4.21×10^{-7}	6.36	4.7×10^{-11}	10.33		
Citric	7.45×10^{-4}	3.13	1.73×10^{-5}	4.76	4.02×10^{-7}	6.40
Glycine	1.66×10^{-10}	9.78				
Lactic	1.37×10^{-4}	3.85				
Phosphoric	0.71×10^{-2}	2.15	6.3×10^{-7}	6.20	4.8×10^{-13}	12.32
Phthalic	1.12×10^{-3}	2.95	3.91×10^{-6}	5.41		
Tartaric	9.3×10^{-4}	3.03	4.30×10^{-5}	4.37		
Base						
	K_b	pK_b				
Ammonia	1.77×10^{-5}	4.75				
Glycine	2.26×10^{-12}	11.65				
Urea	1.5×10^{-14}	13.82				

^a The values given apply only to dilute solutions and are significantly changed in the presence of added salts.

which can combine with nearly all of the added hydrogen ion. There is thus no significant increase in the hydrogen ion concentration of the solution.



Similarly, although added hydroxyl ion neutralizes part of the free hydrogen ion in the solution this loss is immediately made good by further ionization of the undissociated acid, and again there is no appreciable change in the acidity of the solution.



It is the presence of such *buffer mixtures* in living cells which maintains their nearly constant *pH* in the face of fluctuations in their acid content. The capacity of buffers to stabilize *pH* is also of the greatest importance in the laboratory. Experiments with living cells or with the catalysts extracted from living cells are feasible only because they can be conducted in a medium which is buffered against excessive changes in acidity.

The hydrogen ion concentration of a buffer mixture can be calculated from the expression for the dissociation of the weak acid concerned. For example, in a solution containing acetic acid and its sodium salt, the concentration of the acetate ion is essentially that of the salt, since the normally small dissociation of acetic acid is further repressed by the high concentration of acetate ion from the completely ionized salt. For the same reason the concentration of unionized acetic acid is essentially equal to the total concentration of acid present. As long as all concentrations are low, the expression for the dissociation of the weak acid in such a mixture may therefore be written

$$K_a = \frac{[\text{H}^+][\text{salt}]}{[\text{acid}]}$$

The hydrogen ion concentration is thus seen to depend upon the size of the dissociation constant and upon the ratio of acid to salt.

$$[\text{H}^+] = K_a \frac{[\text{acid}]}{[\text{salt}]}$$

This is the standard buffer equation, often used in the form which is known as the *Henderson-Hasselbalch equation*:

$$pH = pK_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

The activity constant of a weak electrolyte varies significantly with the total salt concentration. Thus the constant for aqueous acetic acid is 1.75×10^{-5} , but in 0.1M sodium chloride solution is 2.8×10^{-5} . It is therefore important when the salt concentration is at all high to use the proper dissociation constant in calculating the pH of a buffer mixture.

Preparation of Buffers. The simplest way to prepare a buffer is to put into solution an acid and its salt. For example, suppose that a buffer of pH 5 is to be prepared by adding solid sodium acetate to 0.1M acetic acid. From the buffer equation it is obvious that equal concentrations of salt and acid give a pH equal to the pK_a , or a hydrogen ion concentration equal to the K_a . But 10^{-5} is not equal to the constant for acetic acid (2.8×10^{-5} when the salt concentration is approximately 0.1M) but is roughly one third of that value. This means that the salt concentration must be approximately 3 times the acid concentration to make the pH = 5. For a salt concentration of 0.3M the constant for acetic acid is 2.95×10^{-5} , and this is therefore the appropriate constant to use in calculating the weight of salt which is required.

$$10^{-5} = 2.95 \times 10^{-5} \times \frac{0.1}{x}$$

$$x = \frac{2.95 \times 10^{-6}}{10^{-5}} = 0.295 \text{ moles of sodium acetate}$$

which must be added to 1 liter of 0.1M acid to give the required buffer mixture.

For the preparation of a series of buffers of graded pH it is convenient to begin with a solution of the acid and to form the salt by adding varying amounts of base. A given acid may be used to prepare buffers with a range of pH values from slightly above to slightly below its own pK_a . Obviously the buffering capacity is greatest when the concentrations of salt and acid are equal, and the pH equals the pK_a . Under these conditions the buffer solution is as effective as it can be in preventing pH changes by either added hydrogen or hydroxyl ion. But for limited ranges on either side of this value satisfactory buffers may be prepared. Those in which the concentration of acid is greater than that of salt will be more effective against added base than against acid. When the salt concentration is relatively high this situation is reversed.

Phthalic acid, a dibasic organic acid, is widely used in the preparation of buffers. Since its primary ionization constant is 1.12×10^{-3} , appropriate mixtures of the acid and its monopotassium salt act as buffers with pH values which range from approximately 2.0 to 3.8. Its secondary constant is 3.91×10^{-6} and this means that a series of different mixtures

of the mono- and the dipotassium salts are buffers with pH values which lie between 4.0 and 6.2. In these latter mixtures the "acid" involved is the acid phthalate ion. A few examples will make clear how such solutions are prepared. In the calculations which follow, the solutions are assumed to be relatively dilute and so the salt effect is neglected.

EXAMPLE 1.

To calculate the pH of two different phthalate buffer mixtures.

Suppose that a 0.1M solution of potassium hydrogen phthalate is available. To a liter of this solution 0.05 mole of hydrogen chloride is added. Interaction of the acid phthalate ion with the added hydrogen ion forms approximately 0.05 mole of the unionized phthalic acid. Strictly speaking, as in all buffer mixtures, the actual concentration of unionized acid is somewhat less than that of the added acid, since there is always a slight ionization. But accepting the approximations used in setting up the buffer equation, the solution now contains 0.05 mole of phthalic acid and 0.05 mole of the potassium salt. The hydrogen ion concentration is therefore equal to the primary dissociation constant, 1.12×10^{-3} or the $pH = 3 - 0.05 = 2.95$.

If instead of acid 0.025 mole of potassium hydroxide had been added to 1 liter of potassium acid phthalate solution, the resulting mixture would have contained 0.075 mole of unchanged potassium hydrogen phthalate, and 0.025 mole of the dipotassium salt. In this solution the acid of the buffer mixture is the acid phthalate ion and it is therefore the secondary ionization constant which must be used to calculate the pH .

$$\begin{aligned} [H^+] &= K_2 \frac{[\text{acid}]}{[\text{salt}]} \\ &= 3.91 \times 10^{-6} \times \frac{0.075}{0.025} = 1.17 \times 10^{-5} \\ pH &= 5 - 0.07 = 4.93 \end{aligned}$$

EXAMPLE 2.

Directions for making up buffer mixtures are often given in the following form. To 50 ml. of 0.2M acetic acid add 12.5 ml. of 0.2M NaOH and make the solution to 200 ml. Since it is not the absolute concentrations, but the concentration ratio which determines the pH , the calculation of the resulting pH may use moles per liter or moles per 200 ml., so long as both are in the same units.

There is 0.01 mole of acid in the 50 ml. of solution used. Of this, one quarter is neutralized by base giving 0.0025 mole of salt and leaving 0.0075 mole of the original acid in the final 200 ml. of solution. The pH is therefore

$$\begin{aligned}
 \text{pH} &= 4.76 + \log \frac{0.0025}{0.0075} = 4.76 + \log 1 - \log 3 \\
 &= 4.76 - 0.48 = 4.28
 \end{aligned}$$

EXAMPLE 3.

To prepare a phosphate buffer so that the total phosphate concentration will be 0.1M and the pH = 6.6.

Since the primary dissociation constant for phosphoric acid is 0.71×10^{-2} the mixture cannot consist of the acid and its monopotassium salt, for this would give far too low a pH. But a mixture of mono- and dipotassium phosphate will have as its acid the H_2PO_4^- ion with a dissociation constant of 6.3×10^{-7} and a $\text{pK}_a = 6.20$. These two should therefore be used to prepare a buffer of the desired pH.

Let the required concentration of salt (K_2HPO_4) = x . Since the total phosphate concentration is to be 0.1M, the concentration of acid (H_2PO_4^-) will then be $0.1 - x$.

$$\text{pH} = \text{pK}_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$6.6 = 6.20 + \log \frac{x}{0.1 - x}$$

$$\log \frac{x}{0.1 - x} = 0.4$$

$$\frac{x}{0.1 - x} = 2.51$$

$$x = 0.251 - 2.51x$$

$$3.51x = 0.251$$

$$x = 0.071 \text{ moles per liter of salt (K}_2\text{HPO}_4\text{)}$$

$$0.1 - 0.07 = 0.03 \text{ moles per liter of acid (KH}_2\text{PO}_4\text{)}$$

To prepare 1 liter of this buffer requires

$$0.03 \times 136 = 4.08 \text{ g. KH}_2\text{PO}_4$$

$$0.07 \times 174 = 12.2 \text{ g. K}_2\text{HPO}_4$$

MEASUREMENT OF pH

There are two general methods for the determination of pH, the one colorimetric, the other electrometric.

Colorimetric Methods. The colorimetric estimation of pH is a simple procedure involving comparison of the color of an indicator in the unknown

with the color of the same concentration of this indicator in a buffer of known pH .

The indicators are weak acids or bases, the molecules of which differ in color from the ions which they form in alkaline or acidic solution. A few indicators such as methyl red, methyl orange, and phenolphthalein have ionization constants which cause them to change color at such pH ranges that they can be used to determine the end point of a titration. The indicator chosen for a particular titration must change color at the pH which is reached when an equimolar amount of acid or base has been added. If either the acid or base is a weak electrolyte this pH will be higher or lower than 7, as was indicated on the titration curve for acetic acid.

But there are many more indicators which change color at pH values outside the usual titration ranges. These are suitable for use with a series of buffers to determine the pH value of an unknown solution. In Table 2-IX some of the common indicators are listed with their pH ranges.

TABLE 2-IX. SOME INDICATORS, THEIR RANGES AND COLORS

Indicator	pH Range	Color at Lowest pH	Color at Highest pH	pK_{in}
Thymol blue (acid range)	1.2-2.8	Red	Orange	1.5
Bromphenol blue	3.0-4.6	Yellow	Blue	4.0
Methyl red	4.4-6.2	Red	Yellow	5.1
Bromcresol purple	5.2-6.8	Yellow	Purple	6.3
Bromthymol blue	6.0-7.6	Yellow	Blue	7.0
Phenol red	6.4-8.0	Yellow	Red	7.9
<i>m</i> -Cresol purple (alkaline range)	7.4-9.0	Yellow	Purple	8.2
Thymol blue (alkaline range)	8.0-9.6	Yellow	Blue	8.9
Thymolphthalein	9.3-10.5	Yellow	Blue	10.0

The dissociation constants of these indicator acids are also given as pK values. It should be noted that when an indicator is yellow, for example, at pH 3 and blue at pH 4.6, it will be yellow at all pH values below 3 and blue at those above 4.6. The chief exception is thymol blue which changes from red to yellow between pH 1.2 and 2.8, and from yellow to blue between pH 8.0 and 9.6.

A series of buffers differing by 0.2 pH unit may easily be prepared or purchased. To use these for colorimetric estimation of pH , nine series of buffers of graded pH , corresponding to the nine ranges of pH values given in Table 2-IX for the different indicators, are first set up in a series of tubes. To the tubes containing buffers at pH 1.2, 1.4, 1.6 . . . to 2.8, a definite number of drops of thymol blue is added; bromphenol blue is added to the tubes covering the pH range 3.0 to 4.6; methyl red is put in the tubes of the next series, and so on. In each group of tubes there will result a graduated set of colors which can be matched against the color

obtained when the same indicator is added to the same volume of an unknown solution. There are various laboratory techniques for matching colors as accurately as possible, but the essential operation consists of comparing the color given by the unknown with that given by solutions containing graded ratios of indicator acid and ion.

Electrometric Method. Although for many purposes the colorimetric determination of pH is accurate enough, for experiments in which fine distinctions are important it is necessary to determine this value electrometrically. There are various instruments on the market for doing this, indeed many of them are so adjusted that the estimation of pH consists merely in putting a sample of the solution in the designated cup, turning a knob until the needle points to zero, and reading the pH from a scale. In order to understand why such a procedure gives the information desired, it will be necessary to review briefly some of the facts of electrochemistry.

Electrode Reactions: When a strip of zinc is immersed in a solution of cupric sulfate, metallic copper plates out on the zinc and zinc ions go into solution.



This oxidation-reduction is achieved by a direct transfer of electrons from zinc atom to cupric ion. The fact that such a transfer takes place indicates a tendency on the part of zinc to lose electrons and become an ion, and on the part of the cupric ions to acquire electrons and plate out as metallic copper.

In the galvanic cell these opposite tendencies are utilized to generate an electric current. In a cell the same type of oxidation-reduction reaction takes place, but under such conditions that the electrons must be transferred through an external wire. This stream of moving electrons constitutes an electric current.

A simple galvanic cell is represented diagrammatically in Figure 2.11. One half cell consists of zinc metal dipping into zinc ion, the other of copper dipping into cupric ion. The two metals are connected through the voltmeter and the two solutions through the inverted U-tube filled with sodium chloride solution. When zinc goes into solution as zinc ion the electrons which are left behind on the metal give this electrode a negative charge. When all connections have been made these extra electrons move through the wire and the voltmeter to the copper electrode. Here there is a deficiency of electrons, since the cupric ions tend to acquire them from the metallic copper, and the incoming stream makes good in some measure this loss. The two electrode reactions are



To complete the circuit negative ions move through the salt bridge toward the zinc solution, to balance the charge on the newly formed zinc ions, and positive ions move in the other direction to make up in that half of the cell for the loss of the positive cupric ions. At the moment when the circuit is completed a deflection of the needle of the voltmeter indicates a flow of current, or of electrons. The net chemical result is oxidation

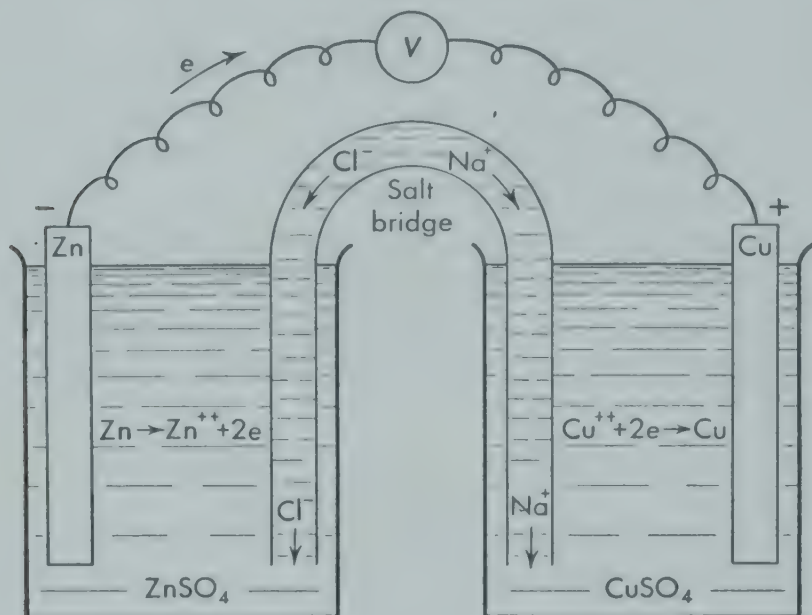


Figure 2.11. Diagram of a voltaic cell.

of the zinc electrode and reduction of the cupric ions. This is expressed as the sum of the two electrode reactions



The actual emf developed by such a cell as has just been described cannot be accurately measured by a voltmeter, since in the process of measurement the voltage gradually changes as the current flows. If we consider the cell reaction above, it is obvious that a process which results in a gradual increase in the concentration of zinc ion, and a decrease in the concentration of cupric ion will ultimately so change the relative concentrations that the reaction will cease. In order to measure the voltage of a galvanic cell it is necessary to use a potentiometer. In this instrument the emf of the cell being measured is exactly balanced by an opposing emf from an outside source, so arranged that it just prevents the flow of current. Under these experimental conditions it is found that a cell of the type described above, set up with molal solutions of zinc salt and of cupric salt, develops an emf of about 1.1 v.

Many different oxidation-reduction reactions can be utilized to generate larger or smaller potential differences. The accepted convention is to

represent such cells with the element-ion pair which develops a negative charge on the left. This means that the pair written on the left is the



one in which oxidation takes place. One of the pair on the right will be reduced. A series of possible cells is indicated below, with the emf which each develops if the solutions used are 1 molal.

Mg		Mg ⁺⁺		Zn ⁺⁺		Zn	1.58 v
Zn		Zn ⁺⁺		Fe ⁺⁺		Fe	0.32 v
Fe		Fe ⁺⁺		Cu ⁺⁺		Cu	0.78 v
Cu		Cu ⁺⁺		2Cl ⁻		Cl ₂ (Pt)	1.02 v

Such a representation of a cell tells us of the first pair, for example, that when magnesium dips into a molal solution of magnesium ions and is

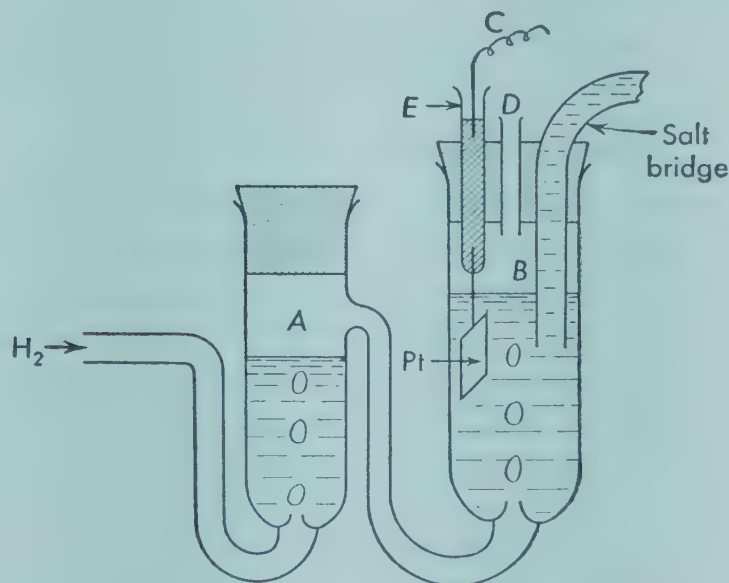


Figure 2.12. Diagram of a gas electrode.

connected through an external wire and a salt bridge (or other device) with zinc dipping into molal zinc ion, there will be a flow of electrons through the wire from the magnesium to the zinc. The magnesium will be oxidized to the ion, and the zinc ion will be reduced to metallic zinc. It should be noted that the voltage developed by the (Zn:Fe) pair plus that of the (Fe:Cu) pair equals that previously noted for the (Zn:Cu) cell. •

$$\text{emf (Zn:Fe)} + \text{emf (Fe:Cu)} = \text{emf (Zn:Cu)}$$

$$0.32 \quad + \quad 0.78 \quad = \quad 1.10 \text{ v}$$

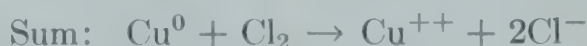
As the last cell in the list would indicate, electrodes may be gaseous as well as metallic. One form of gas electrode is shown diagrammatically in Figure 2.12. Hydrogen gas is bubbled through the cell solution in

chamber *A* to saturate it with water. In chamber *B* a platinum electrode coated with platinum black dips into the same cell solution and is connected to the outside circuit (*C*) through the mercury which fills the tube *E*. On the catalytically active spongy platinum, equilibrium is rapidly established between the hydrogen ion in solution and the gas, excess



of which escapes at *D*. To use such an electrode as part of a galvanic cell the vessel *B* must be connected through a salt bridge or a liquid junction with the solution of another half cell, while the outside wire *C* is connected to the other electrode.

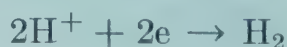
If such an arrangement were used to set up the (Cu:Cl) cell it would be found that the copper furnishes electrons for the reduction of the chlorine, and that it is itself oxidized to cupric ion. The electrode reactions for this combination are



Thus in this cell copper is oxidized although when this same copper half cell is combined with a zinc half cell cupric ions are reduced. Evidently the "tendency" to be oxidized or reduced is not absolute, but relative, and the direction in which the oxidation-reduction will go depends on which of the two elements concerned has the greater electronegativity, or tendency to acquire electrons. In order to obtain a flow of electrons from one electrode to another there must be a difference in this tendency and the larger the difference, the larger the emf which the cell develops. For example, magnesium is strongly electropositive and chlorine strongly electronegative. A (Mg:Cl) cell registers this difference as a high emf. On the other hand a cell made up of tin and copper and their ions has a much smaller emf, indicating that the tendency of tin to form stannous ions is not appreciably different from that of copper to form cupric ions. As a result the cupric ions do not draw any great number of electrons from the tin. We can measure with a potentiometer this *difference* in potential between two element-ion pairs, but there is no way to measure directly the individual "tendencies" on which the difference depends. However, by comparing all other elements with hydrogen it has been possible to construct a table of relative values, from which the difference between any two can be computed.

The reference electrode, to which all others are compared, is the standard hydrogen half cell consisting of hydrogen gas at 1 atmosphere pressure bubbling around a platinum electrode which dips into a solution in which the effective hydrogen ion concentration is 1 molal. The emf developed

when this half cell is combined with a zinc electrode in a solution 1 molal in zinc ion is 0.761 volts, and the flow of electrons is from the zinc to hydrogen. For the purposes of constructing a scale, the electrode potential of the standard hydrogen half cell is arbitrarily set at zero, thus making the potential of the standard zinc electrode (zinc dipping into molal zinc ion) -0.761 v. The negative sign indicates that the zinc electrode is the negative one, that is, that in this particular cell zinc is oxidized and hydrogen ion reduced.



When this same hydrogen half cell is combined with a standard copper half cell (copper metal and molal cupric ion), the emf of the cell proves to be 0.34 v. But in this cell oxidation takes place at the hydrogen electrode, which is therefore the negative electrode. The potential of the standard copper half cell is therefore $+0.34$ v.

TABLE 2-X. STANDARD REDUCTION POTENTIALS

Electrode Reaction	Electrode	E^0 (volts)
$\text{Na}^+ + e = \text{Na}$	Na^+, Na	-2.71
$\text{Mg}^{++} + 2e = \text{Mg}$	$\text{Mg}^{++}, \text{Mg}$	-2.34
$\text{Zn}^{++} + 2e = \text{Zn}$	$\text{Zn}^{++}, \text{Zn}$	-0.76
$\text{Fe}^{++} + 2e = \text{Fe}$	$\text{Fe}^{++}, \text{Fe}$	-0.44
$\text{Cr}^{+++} + e = \text{Cr}^{++}$	$\text{Cr}^{+++}, \text{Cr}^{++} (\text{Pt})$	-0.40
$\text{Sn}^{++} + 2e = \text{Sn}$	$\text{Sn}^{++}, \text{Sn}$	-0.14
$2\text{H}^+ + 2e = \text{H}_2$	$\text{H}^+, \text{H}_2 (\text{Pt})$	0.00
$\text{Cu}^{++} + 2e = \text{Cu}$	$\text{Cu}^{++}, \text{Cu}$	$+0.34$
$\text{Fe}^{+++} + e = \text{Fe}^{++}$	$\text{Fe}^{+++}, \text{Fe}^{++} (\text{Pt})$	$+0.77$
$\text{Ag}^+ + e = \text{Ag}$	Ag^+, Ag	$+0.80$
$\text{Cl}_2 + 2e = 2\text{Cl}^-$	$\text{Cl}_2, \text{Cl}^- (\text{Pt})$	$+1.36$
$\text{Co}^{+++} + e = \text{Co}^{++}$	$\text{Co}^{+++}, \text{Co}^{++} (\text{Pt})$	$+1.82$

In Table 2-X is given a list of a few standard electrode potentials (E^0) obtained in this way. Note that in a standard half cell the ion is always present in molal concentration and any gas involved is used at 1 atmosphere pressure. When the potential given in the table carries a negative sign it indicates that the electrode reaction which as written is a reduction, actually tends to go in the opposite direction and to result in an oxidation. Similarly a positive voltage means that the reaction tends to take place spontaneously as written. Thus when any two half cells are combined, the one with the more negative potential becomes the one in which oxidation takes place, while the one with the more positive potential undergoes a reduction.

The emf to be obtained from any combination of standard half cells depends upon the difference between their individual electrode potentials. These potentials as given in the table are reduction potentials, that is they measure the tendency of the element in question to be reduced. Using this set of values the emf of the cell may be calculated by subtracting the reduction potential of the anode from that of the cathode. The anode is defined as the electrode at which oxidation takes place, and the cathode as the electrode at which reduction takes place. Thus in a voltaic cell the cathode is *positively* charged and the anode *negatively* charged. The opposite is true when the electrodes are being used for electrolysis.

$$E_{\text{cell}} = E_{\text{cathode}}^0 - E_{\text{anode}}^0$$

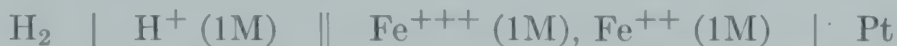
For the (Zn:Cu) cell this means

$$\begin{aligned} E_{\text{cell}} &= E(\text{Cu}^{++} \rightarrow \text{Cu}) - E(\text{Zn} \rightarrow \text{Zn}^{++}) \\ &= +0.34 \quad \quad \quad - (-0.76) \\ &= 1.1 \text{ v} \end{aligned}$$

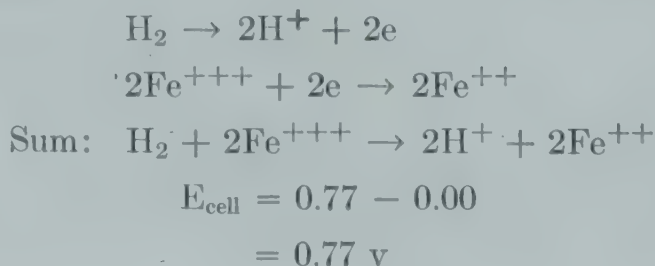
In a cell made up of standard silver and copper half cells the copper will be oxidized since it has the lower reduction potential, and the silver ion will be reduced.

$$\begin{aligned} E_{\text{cell}} &= E(\text{Ag}^+ \rightarrow \text{Ag}) - E(\text{Cu} \rightarrow \text{Cu}^{++}) \\ &= +0.80 \quad \quad \quad - (+0.34) \\ &= 0.46 \text{ v} \end{aligned}$$

Scattered through the table are certain electrode reactions involving two ions instead of an element-ion pair. It might be expected that if a cell were set up in which electrons could move through a wire to a platinum electrode dipping into a mixture of ferric and ferrous ions, the ferric ions would be reduced. The positive sign in the table for the reaction $\text{Fe}^{+++} + e \rightarrow \text{Fe}^{++}$ indicates that this is true, and that ferric ions do in fact take up electrons readily. Thus in a cell made up with the standard hydrogen half cell combined with an inert electrode dipping into a solution .1 molal in ferric and ferrous ions, the hydrogen is oxidized.



The reactions and voltages are computed as with other cells. The electrode reactions are



Effect of Concentration: Since all the electrode reactions are reversible it is obvious that the emf of a galvanic cell depends not only upon the element-ion pairs which are used, but upon the concentrations of the solutions.

The relation between the electrode potential of a standard half cell and that of the same element in a solution other than molal was developed theoretically by Walther Nernst (1864–1941) about 1889. It is expressed in the equation

$$E = E^0 \pm \frac{1.98 \times 10^{-4} T}{n} \log C$$

in which E denotes the new electrode potential in volts, E^0 is the standard electrode potential, T denotes the Kelvin temperature, n is the valence of the element, and C is the effective molal concentration of the ion. For the solutions with which we deal C may be taken as the molar concentration of the ion. In using the equation the positive sign is used when the ion concerned carries a positive charge, and the negative sign for a negative ion. At 25°C. the equation becomes

$$E = E^0 \pm \frac{0.0592}{n} \log C$$

Consider a half cell in which zinc is in contact with 0.001M zinc sulfate solution. Here $n = 2$, the ion carries a positive charge, and the electrode potential becomes

$$\begin{aligned} E &= -0.76 + \frac{0.0592}{2} \log 10^{-3} \\ &= -0.76 + (0.0296)(-3) \\ &= -0.76 - 0.089 \\ &= -0.849 \text{ v} = -0.85 \text{ v} \end{aligned}$$

Thus decrease in the concentration of zinc ion, by making it easier for ions to form, has resulted in more rapid formation of new ions, which means a more negative potential for the electrode.

With a chlorine electrode and a solution 0.001M in chloride ion, $n = 1$ and the Nernst equation is used with a negative sign.

$$\begin{aligned} E &= +1.36 - \frac{0.0592}{1} \log 10^{-3} \\ &= +1.36 - (0.0592)(-3) \\ &= +1.36 + 0.178 \\ &= +1.538 \text{ v} = +1.54 \text{ v} \end{aligned}$$

With the chlorine electrode a decrease in the concentration of the negative ion has made the electrode more positive since it makes it easier for the element to go into solution as a negative ion.

When the half cell consists of an oxidation-reduction pair and an inert electrode, the equation for calculating the potential for other than standard concentrations becomes

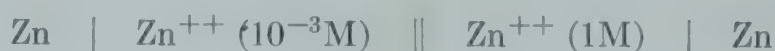
$$E = E^0 + \frac{0.0592}{n} \log \frac{[\text{oxidized}]}{[\text{reduced}]}$$

where n stands for the number of electrons gained in the reduction. The final potential is thus seen to depend not on the absolute concentrations of the ions, but on their ratio.

For the electrode reaction $\text{Co}^{+++} + e \rightarrow \text{Co}^{++}$, the standard electrode potential is +1.82 v. If the concentration of the cobaltic ion is made 100 times that of the cobaltous the electrode potential becomes

$$\begin{aligned} E &= 1.82 + \frac{0.0592}{1} \log 100 \\ &= 1.82 + (0.0592)(2) \\ &= 1.94 \text{ v} \end{aligned}$$

Concentration Cells: Since there is a potential difference between electrodes of the same element dipping into solutions of different ionic strengths, it is possible to use two such element-ion pairs to make a cell. For example a zinc cell could be set up as follows:



Calculation of the voltage is carried out as before by subtracting the potential of the anode from that of the cathode.

$$\begin{aligned} E_{\text{cell}} &= -0.76 - (-0.85) \\ &= 0.09 \text{ v} \end{aligned}$$

The emf of a concentration cell may be expressed in general terms as

$$\begin{aligned} E &= (E \text{ for the cathode}) - (E \text{ for the anode}) \\ &= \left(E^0 + \frac{0.0592}{n} \log C_2 \right) - \left(E^0 + \frac{0.0592}{n} \log C_1 \right) \end{aligned}$$

where C_1 is the concentration of the more dilute of the two solutions of a positive ion.

$$E = E^0 - E^0 + \frac{0.0592}{n} (\log C_2 - \log C_1)$$

$$= \frac{0.0592}{n} \log \frac{C_2}{C_1}$$

A simple hydrogen concentration cell is the basis for many *pH* meters. It may be represented as



Since in this arrangement C_2 is molar, and n is also equal to 1, the expression for calculating the voltage of the cell reduces to

$$E = \frac{0.0592}{1} \log \frac{1}{[\text{H}^+]}$$

where $[\text{H}^+]$ represents the hydrogen ion concentration of the unknown solution. But $\log \frac{1}{[\text{H}^+]}$ is the *pH*, hence

$$E = 0.0592 \times \text{pH}$$

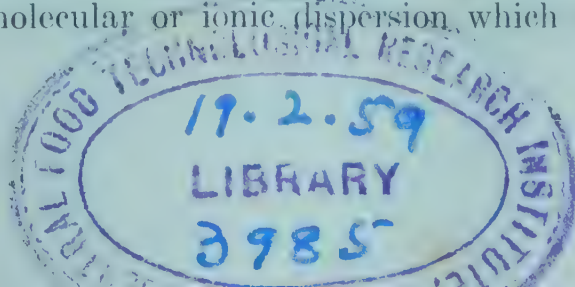
and the *pH* of an unknown solution measured in such a concentration cell as has been described would be numerically equal to the measured emf of the cell divided by 0.0592.

In practice modern *pH* meters seldom use hydrogen electrodes, but whether the reference electrode is hydrogen or the glass electrode or the quinhydrone electrode the operative principle is a variant of the one just outlined.

The Colloidal State

It has been estimated that about 90 per cent of the organic matter in many tissues occurs there in a special state of subdivision, the colloidal state, characterized by large surface areas. The word colloid (Greek *kolla* = glue) was coined by Thomas Graham (1805–1869) to distinguish those substances, such as starch and gelatin, which do not pass through a parchment paper membrane separating their solution from pure water, from those which do pass through. The latter he called *crystalloids*, since many of them crystallize readily.

Since Graham's time some substances apparently inherently colloidal in nature have been obtained in crystalline form, and most chemists have found to their sorrow that many which are normally crystalline do occasionally form colloidal solutions. Accordingly it is now recognized that the unique properties associated with the colloidal state depend not upon the character of the solute but upon its specific state of subdivision. This is intermediate between the molecular or ionic dispersion, which obtains



in a true solution and the particle size which is found in fine suspensions and is visible under a microscope. Although a very precise definition of colloidal size is impossible, Table 2-XI gives a classification of particle sizes which indicates roughly what is meant by colloidal dimensions.

TABLE 2-XI. PARTICLE SIZES ^a

0.1 m μ	1 m μ	0.01 μ	0.1 μ	1 μ	10 μ	100 μ	1 mm.
True Solutions :		Colloidal Solutions :		Suspensions			
Invisible	Ultramicroscopic			Microscopic			
Pass filters	Pass filters			Do not pass filters			
Dialyze	Do not dialyze			Do not dialyze			
Large osmotic effect	Small osmotic effect			No osmotic effect			

^a The dimensions are not on a linear but on a logarithmic scale.

In a colloidal system one substance in a fine state of subdivision is evenly dispersed throughout a second. The first is known as the *dispersed* or *discontinuous phase*, the other as the *dispersion medium*, or the *continuous phase*. In biological systems the dispersion medium is usually water, although under some circumstances water may constitute the dispersed phase. (See later discussion of emulsions.)

The properties of colloidal systems depend very little on the chemical properties of the components, but are chiefly referable to the large surface area between the two phases. If a cube 1 cm. on a side were subdivided into cubes 10 m μ (10⁻⁶ cm.) on a side, the resulting particles would be of colloidal dimensions and the surface area would have increased from 6 square cm. to 60 square meters. In the large interfacial area between these minute particles and a dispersion medium, surface equilibria are set up similar to those previously discussed in general terms. The molecules of the solvent form surface films around each particle similar to those at a water/air interface. Since the interfacial tension thus generated tends, as free energy always does, to be reduced, any surface active molecules in the dispersion medium become concentrated in the interfacial film. If there are electrolytes also in the solution the particles of the colloid may become charged through preferential adsorption of one ion at the interface.

Three major types of colloidal system are known as *sols*, *gels*, and *emulsions*. A sol consists of a solid phase dispersed in a liquid to yield a mobile, fluid colloidal system; when the resulting colloid has a firm, jelly-like texture it is known as a gel. An emulsion is a colloidal dispersion of one liquid in another.

SOLS

Colloidal particles are too small to be visible under an ordinary microscope. They are, however, visible in the ultramicroscope for the same reason that dust particles can be seen where a fine beam of light penetrates a darkened room. The particles of dust reflect and scatter the light waves, giving rise to a diffused light in which the dust is seen. In the ultramicroscope a beam of light is viewed at right angles through a microscope. When the light is directed upon a true solution the path of the beam is invisible since the particles are too small to scatter the light. The field of the microscope appears dark. But when the light shines into a colloidal solution a tiny point of light appears against the dark background wherever the beam impinges upon a particle large enough to diffract it. The points of light move about in a random fashion because the colloidal particles themselves are in a disordered kind of motion as a result of bombardment by the molecules of the dispersion medium. This motion, known as Brownian movement, was first noted by the Scots botanist Robert Brown as he examined suspensions of tiny pollen grains under the microscope.

The particles thus revealed in colloidal sols may belong to either of two classes, depending upon whether they have or have not an attraction for the dispersion medium. These classes are *lyophilic* or solvent-loving sols, and *lyophobic* or solvent-hating sols. Since in biological systems the dispersion medium is water the groups are also referred to as hydrophilic and hydrophobic.

Hydrophobic Sols. Many substances which have no attraction for water, and consequently no tendency to form true solutions, can be induced to form colloidal sols which are clear and transparent and often quite beautifully colored. Among these are the noble metals, silver, gold, and platinum, some metal sulfides including arsenic and antimony trisulfides, ferric and aluminum hydroxides, and a number of organic dyes. In these sols groups of atoms or molecules are held together in particles of colloidal dimensions stabilized by electric charges of the same sign. A typical hydrophobic sol exhibits the following properties:

1. In viscosity and surface tension it differs very little from pure water.
2. Its particles are electrically charged as shown by their migration in an electric field. The source of the charge varies from one sol to another. Colloidal ferric hydroxide may carry a positive charge because of dissociation of hydroxyl ions from the surface of the particle. Or it may, like many other colloidal particles, acquire a charge by capture of ions. A given colloid adsorbs preferentially either negative or positive ions, so that particles of ferric and aluminum hydroxides, for example, are always positively charged, while the colloidal metals carry negative

charges. The stability of hydrophobic sols depends largely upon these charges which prevent the particles from coalescing into aggregates too large to remain dispersed.

3. Hydrophobic sols are very sensitive to small amounts of added electrolyte, which induce flocculation of the colloid. This is apparently due to neutralization of the charge on the colloidal particles by the charge on ions of the opposite sign. Thus the negative arsenious sulfide is precipitated by much lower molar concentrations of trivalent cations than of monovalent ones, while the positive colloidal ferric hydroxide is coagulated much more effectively by trivalent anions than by monovalent.
4. Hydrophobic sols are *irreversible*, that is, once they have been precipitated it is not possible to redisperse them by removing the precipitating agent.

Hydrophilic Sols. Most of the colloids which are important in biological systems are hydrophilic. These include the large group of proteins, the phospholipids, and many plant and animal polysaccharides. It is probable that the protein and polysaccharide particles consist of single giant molecules stabilized by hydration. In contrast with the hydrophobic colloids the water-loving sols exhibit the following properties:

1. Their solutions have greater viscosity and lower surface tension than pure water.
2. Although their particles are usually charged, it is not the charges alone which confer stability upon the sols. The colloidal particles are also heavily hydrated, and it is the presence of this protective layer of adsorbed solvent which prevents the particles from coalescing to form a precipitate.
3. The sign of the charge carried by a given colloid is not specific but may change with a change in pH. Thus the same protein may carry a net positive charge in neutral solution and a net negative charge in basic solution. Furthermore it can undergo a change from one charge to the other without precipitating, though at the midpoint, when its net charge is zero, it is particularly easy to precipitate.
4. The hydrophilic sols are more stable than the hydrophobic to added *low* concentrations of electrolyte. This lack of sensitivity probably results from the fact that the stability of these sols depends more on the water envelope and less on the repulsion of like charges.
5. When certain electrolytes are added in *high* concentration even the hydrophilic sols precipitate. This process is known as *salting out* and is carried out by saturating or half saturating the colloid solution with such a salt as ammonium sulfate or sodium sulfate. It is believed that the added salt competes with the colloid for water and that the precipi-

tation occurs when the aqueous envelope around the colloidal particles has been removed.

5. A hydrophilic sol is a *reversible* colloid, since removal of the electrolyte which has been used to salt it out allows the precipitate to revert to colloidal form. This may be achieved by immersing the precipitate, enclosed in a semipermeable sac, in a large volume of water or in running water. The small electrolyte ions of the precipitant dialyze away, but the larger colloidal particles are retained and gradually go back into colloidal solution as the electrolyte concentration is reduced.

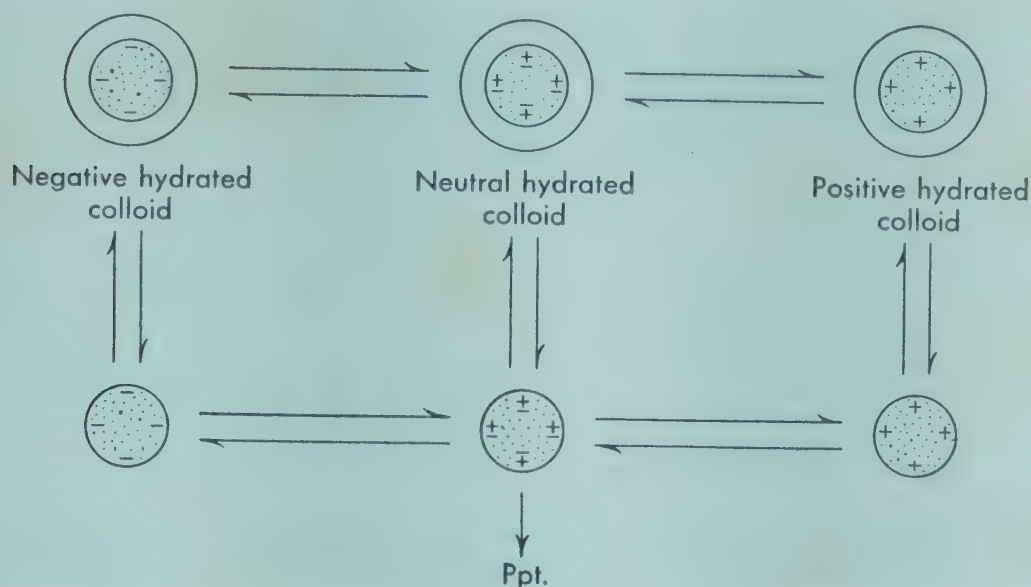


Figure 2.13. Diagrammatic representation of various types of colloidal particles. Those in the upper row are typical lyophilic particles; those below are lyophobic.

A conventional representation of various colloidal particles and their relationship to each other is given in Figure 2.13.

Interaction of Two Colloidal Sols. *Protective Colloids:* The greater stability of hydrophilic sols may be used to confer stability on the more sensitive hydrophobic colloids. For example, small amounts of various plant gums added to inorganic sols reduce the likelihood of precipitation, probably through the formation of a thin protective coating. This property of hydrophilic colloids finds a diagnostic use in the clinical test known as the "gold number." The gold number is defined as the number of milligrams of hydrophilic colloid which just fails to prevent the change in color of a standard gold sol when 1 ml. of 10 per cent sodium chloride is added to 10 ml. of the sol. In certain diseases of the central nervous system the amount of protein (hydrophilic colloid) in the spinal fluid varies from the normal, giving rise to abnormal gold numbers. Comparison of the gold number of the fluid from a patient with that of normal spinal fluid is often an aid in diagnosing the type of nervous disease.

Sensitization: While the presence of hydrophilic colloid may confer stability upon the more sensitive hydrophobic sols, this occurs only when

the concentration of the protecting sol is above a certain critical level. Addition of a hydrophilic sol in lower concentration than this causes the hydrophobic sol to be less stable than usual and hastens its flocculation. This is believed to depend upon the ability of the few hydrophilic particles to catch and hold together a number of hydrophobic units. These relatively large aggregates are then very easily precipitated by electrolytes. The difference between protection and sensitization is indicated diagrammatically in Figure 2.14.

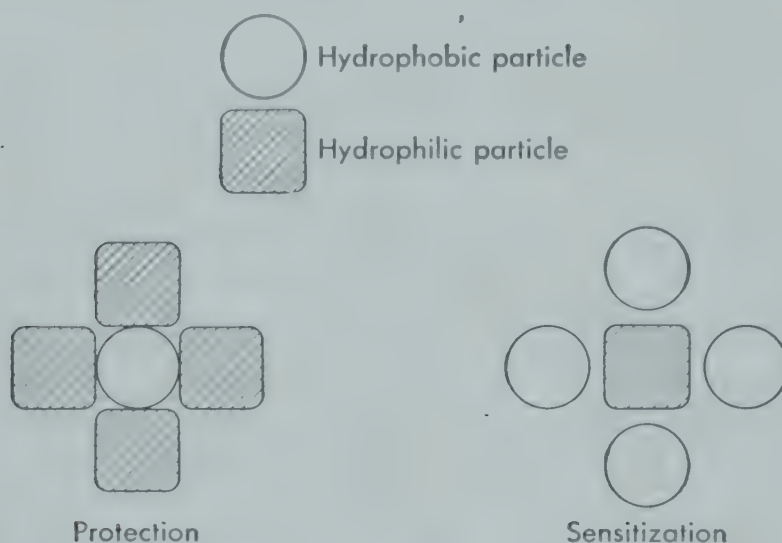


Figure 2.14. Diagrammatic representation of the aggregates which form when hydrophilic colloids protect or sensitize a hydrophobic colloid.

Coacervation: When two hydrophilic colloids of opposite charge are mixed, it may happen that the dispersed phases unite to form a complex which first separates as small liquid drops. These then coalesce to form a viscous liquid layer, known as a *coacervate* (*acervus* = heap). That the oppositely charged particles retain their identity and their charges can be shown by placing the coacervate in an electric field. The oppositely charged particles are then found to move away from the viscous coacervate toward the appropriate electrodes. The stability of the coacervates is believed to result from a balance between the electrostatic attraction of the charged particles and the effect of the water envelope which prevents their approaching each other closely enough to achieve a neutralization of the opposite charges. It has been suggested that cell membranes may prove to be coacervate films, made from some of the oppositely charged colloids known to be present in the cell contents.

GELS

The name *gel* is given to a lyophilic colloid which is characterized by a somewhat elastic rigidity. This semi-solid texture is believed to result from a tendency on the part of some colloidal particles to remain united

when suitable groups come into contact, thus forming what is sometimes described as a "brush heap" structure. As this simile would suggest, the units in the aggregates are somewhat linear in nature and form interlacing masses. Such a structure is indicated diagrammatically in Figure 2.15.

Gelatin is a familiar example of a substance which forms a gel, and the ease with which its gel can be liquefied by heat illustrates the reversibility of the sol \leftrightarrow gel transformation. Whether a gelatin solution will take the form of a sol or a gel depends upon the concentration of the gelatin, the temperature, and the pH.

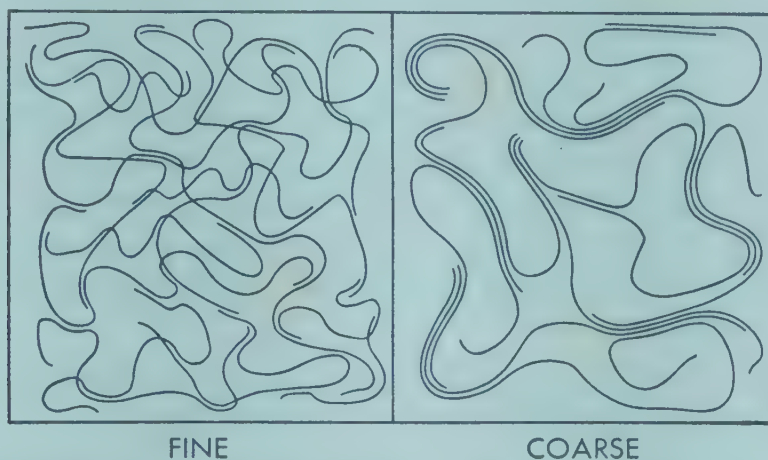


Figure 2.15. Schematic diagrams of gel structure. In the fine network the long macromolecules associate with others here and there for short distances; in the coarse network each aligns itself with a few others for long distances. (From J. D. Ferry, "Protein Gels," *Adv. in Prot. Chem.*, 4:1, 1948.)

The clotting of blood is a biological example of gel formation. In the plasma are a number of proteins in solution, including the one known as fibrinogen. At a point of injury this substance is somewhat changed to become fibrin, which then forms a local gel or clot.

One of the most striking properties of the gels is their ability to take up water. This *imbibition* as it is called is often accompanied by extreme examples of swelling. It is of biological interest that the extent of the swelling is dependent upon the ions which are present. With some colloids pH is the determining factor; with others there is evidence of ionic antagonisms, some ions promoting swelling, others preventing it. This is one of many examples of the antagonistic biological effects of different ions. Often the antagonism is between monovalent and divalent ions, but sometimes it is between two of the same valence. For example, when the blood of an experimental animal is entirely replaced by a solution of pure salts, the heart will continue to beat provided the solution is isosmotic with blood and provided further that it contains a suitable ratio of sodium, calcium, and potassium ions. But even with the correct osmotic pressure, if only one type of cation is used the heart beat gradually diminishes and finally ceases entirely. In another type of experiment it

has been found that raising the concentration of magnesium in the plasma from its normal value of 2-3 mg. per 100 ml. to about 20 mg. results in unconsciousness. But if there follows injection of a corresponding amount of calcium ion this effect is entirely and rapidly reversed. There is at the moment no way of knowing how either of these biological effects is mediated, but it is at least suggestive that simple colloids are sensitive in much the same selective way to varying ionic ratios.

Some gels contract on standing and squeeze out part of the fluid caught in the network of colloidal particles. This process is known as *syneresis*, and is responsible for the slow separation of serum from a blood clot.

EMULSIONS

At the surface of contact between two immiscible liquids there is set up an interfacial tension. When two such liquids are shaken together violently one may form droplets in the other, but on standing the droplets coalesce and the liquids separate. This is to be expected, since droplet formation has greatly increased the surface and therefore the free surface energy of the system.

To convert such a temporary emulsion into a permanent one it is necessary to reduce the interfacial tension. This is usually done by adding something which acts as an emulsifier. The most familiar emulsifying agent is soap which lowers the interfacial tension between water and insoluble fatty material and so promotes the emulsification of the fat.

When both phases of a colloidal system are liquid, either one may become the dispersed phase. Thus an oil:water emulsion may consist of oil droplets in a continuous aqueous medium, or the water may be dispersed as droplets in a continuous oily phase. The form taken by any particular pair depends upon the emulsifying agent used. Thus when this agent is sodium oleate it favors formation of oil-in-water emulsions, but calcium oleate stabilizes an emulsion of the water-in-oil type. This is usually explained by picturing the stabilizer, which concentrates in a film at the interface, as actually a third phase separating the two liquids. Then if the interfacial tension between water and the film is lower than that between oil and the film, the film reduces its area on the oily side by formation of oily droplets in the water. When the interfacial tension between water and the film is the greater of the two the reverse occurs and water droplets are dispersed in the oil.

In this connection it is interesting to note that emulsifying agents of both sorts are almost universally distributed in living cells. This makes it easy to understand how a cell membrane could change its character from time to time and so be permeable first to one type of solute and then to another. If this membrane consists in part at least of fatty material

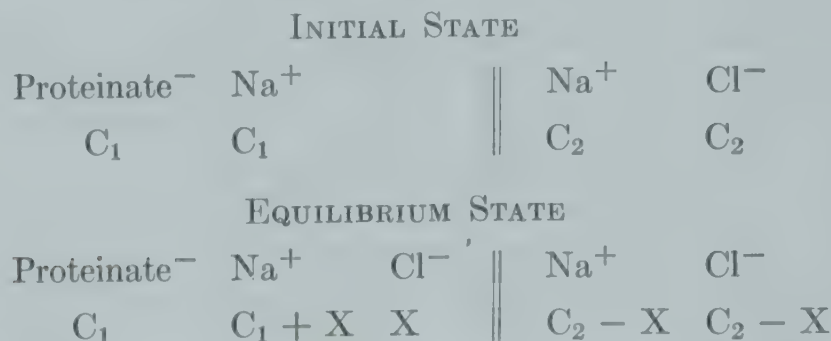
this might be present at a given moment as an oil-in-water emulsion, stabilized both by adjacent sodium ions and by phospholipids of which the surface active lecithin is the most abundant. But cells also contain two substances which favor formation of a water-in-oil dispersion, namely calcium ions and a complex organic substance, cholesterol, which has many properties which are the opposite of those of lecithin. If calcium ion or cholesterol or both were to move to the cell surface there would result a reversal of phase in the membrane, thus making the oil the continuous phase with watery droplets dispersed through it. During the time when the water was continuous throughout the film, water-soluble molecules and ions could pass the cell membrane by dissolving in one side and diffusing through. Later when the continuous phase had become oily, fat-soluble molecules would be admitted to the cell by a similar process. It should be noted that emulsification furnishes another example of ion antagonism, the monovalent ion predisposing to one type of organization and the divalent to the other. It seems that the plan of governing by a system of "checks and balances" was invented by living cells long before our lawmakers appropriated it.

THE DONNAN EQUILIBRIUM

Although particles of colloidal size cannot themselves diffuse through a semipermeable membrane, the presence of these large charged particles on one side of such a membrane does markedly influence the distribution of ions which are able to diffuse. This may at first sight seem surprising. Thus if a protein solution in a collodion sac were to be immersed in a solution of sodium chloride it might seem reasonable to expect that the sodium and chloride ions would ultimately distribute themselves evenly on both sides of the membrane, which is freely permeable to them and impermeable to the colloid. However this proves not to be the case. In the equilibrium which is actually established the ionic concentrations on the two sides of the membrane are unequal and the extent of the inequality depends on the relative concentrations of colloid and ions. This phenomenon was first studied quantitatively by F. G. Donnan of University College, London, about 1911, and has come to be known by his name. As an example of a Donnan equilibrium, consider two chambers separated by a semipermeable membrane. In the one is a solution of a protein which will be assumed for the sake of simplicity to give rise to one sodium ion and a singly charged protein anion. On the other side of the membrane is a solution of sodium chloride to which the membrane is freely permeable. Donnan showed that in such a system ions migrate into the colloid chamber until at equilibrium the ion concentrations satisfy the expression

$$[\text{Na}^+]_1[\text{Cl}^-]_1 = [\text{Na}^+]_2[\text{Cl}^-]_2$$

in which the subscripts refer to the two sides of the membrane. The initial and final states are shown in the diagram which is adapted from a similar chart in Donnan's original paper.



In order to maintain electrical neutrality, equal numbers of sodium and chloride ions must move across the membrane, thus causing the sodium ion concentration on the colloid side to be greater than that of the chloride ion. At equilibrium the Donnan equation reads

$$(C_1 + X)X = (C_2 - X)^2$$

This means that the concentration of sodium ion inside the colloid compartment must be larger than its concentration outside, and that the chloride concentration is correspondingly higher outside than in.

$$\frac{[Na^+]_1}{[Na^+]_2} = \frac{[Cl^-]_2}{[Cl^-]_1}$$

Thus when in the initial state the concentration of protein is, for example, ten times that of sodium chloride, the final concentration of sodium ion in the colloid compartment becomes approximately eleven times that of the sodium ion outside, with the chloride ion concentrations unequal in the reverse sense. The figures given in Table 2-XII show the

TABLE 2-XII. DONNAN EQUILIBRIA WHEN ONE ION IS COMMON TO BOTH CHAMBERS ^a

Original Concen- tration Protein	Original Concen- tration NaCl	Equilibrium Conc. in Colloid Chamber		Equilibrium Conc. Outside Colloid Chamber	
		Na ⁺	Cl ⁻	Na ⁺	Cl ⁻
C ₁	C ₂	C ₁ + X	X	C ₂ - X	C ₂ - X
0.1	1.0	0.576	0.476	0.524	0.524
1.0	1.0	1.33	0.33	0.66	0.66
1.0	0.1	1.008	0.0083	0.092	0.092

^a Adapted from figures in F. G. Donnan, *Z. Electrochem.*, 17:572, 1911.

relation between the initial and the final states for various concentration ratios.

The situation is similar when no common ion is involved. In Table 2-XIII are tabulated the data from an experiment in which the dye Congo

TABLE 2-XIII. DONNAN EQUILIBRIA WITH NO COMMON ION ^a

Original Concen- tration NaR	Original Concen- tration KCl	Equilibrium Conc. Inside Chamber			Equilibrium Conc. Outside Chamber		
		Na ⁺	K ⁺	Cl ⁻	Na ⁺	K ⁺	Cl ⁻
C ₁	C ₂	C ₁ - Z	X	Y	Z	C ₂ - X	C ₂ - Y
100	1.0	99	0.99	0.01	1.0	0.01	0.99
10	1.0	9.2	0.90	0.10	0.8	0.1	0.9
1	1.0	0.66	0.66	0.33	0.33	0.33	0.66

^a Adapted from figures in F. G. Donnan, *Z. Electrochem.*, 17:572, 1911.

red (NaR) which furnishes sodium ion and a negative colloidal ion R⁻ was separated by a membrane from solutions of potassium chloride. In the final distribution both cation concentrations were higher in the colloid compartment, with a corresponding but opposite disparity in the anion concentrations.

Results such as these make it easier to understand how most of the potassium in the blood stream can be concentrated inside the red blood cells and how a marine alga (*Valonia*) is able to maintain in its protoplasm a far lower sodium ion and a far higher potassium ion concentration than obtains in the sea water which bathes it.

Suggestions for Further Reading

GENERAL

BULL, H. B., *Physical Biochemistry*, 2nd ed., Wiley, New York, 1951.
CHAPIN, W. H., *Second Year College Chemistry*, 5th ed. revised by Steiner, L. E., Wiley, New York, 1943.
CLARK, W. M., *The Determination of Hydrogen Ions*, Williams and Wilkins, Baltimore, 1928.

This is the classical book on the subject and while some of it is out of date it is still well worth reading.

MATSEN, F. A., MYERS, J., and HACKERMAN, N., *Pre-Medical Physical Chemistry*, Macmillan, New York, 1949.
MELDRUM, W. B., and GUCKER, F. T., *Introduction to Theoretical Chemistry*, American Book, New York, 1936.

This book is written with grace and charm as well as clarity, and with an historical perspective which adds greatly to its value.

SMALL, J., *pH and Plants*, Baillière, Tindall and Cox, London, 1946.

COLLOIDS

McBAIN, J. W., *Colloid Science*, Heath, New York, 1950.

LIGHT ABSORPTION

BARNES, R. B., LIDDEL, U., and WILLIAMS, V. Z., "Infrared Spectroscopy," *Ind. and Eng. Chem., Anal. Ed.*, 15:659, 1943.

This paper gives an excellent summary as well as a great number of reference curves.

BRODE, W. R., *Chemical Spectroscopy*, Wiley, New York, 1943.

HARRISON, G. R., LORD, R. C., and LOOFBOUROW, J. R., *Practical Spectroscopy*, Prentice-Hall, New York, 1948.

KLOTZ, I. M., and GRISWOLD, P., "Infrared Spectra and the Amide Linkage in a Native Globular Protein," *Science*, 109:309, 1949.

STEVENS, P. G., and SPALDING, S. C., JR., "A New Synthesis of the Cyclopentane Ring," *J.A.C.S.*, 71:1687, 1949.

In this paper infrared spectroscopy is used to prove the identity of two compounds.

WEISSBERGER, A. (ed.), *Physical Methods of Organic Chemistry*, Interscience, New York, 1949, Chapters 21 and 22.

Study Questions

1. How is the solubility of a gas in water affected by changes in temperature? in pressure?

2. Define the "absorption coefficient" of a gas.

3. Calculate the weight of carbon dioxide which would be dissolved by 2 liters of water at 35°C.

4. What is meant by plasmolysis? How is it brought about and how may it be reversed?

5. Why is 0.85 per cent sodium chloride solution called "physiological saline"? What is the molarity of this solution? How could you prepare a solution of glucose which would be approximately isosmotic with it?

6. Sketch an imaginary absorption curve, indicating the various units in which the wave lengths of the light might be plotted. In what units might the absorptions be plotted? How would the same curve differ from the one sketched if it were plotted in terms of transmittancy?

7. What is the special importance of infrared absorption curves?

8. What is the pH of a solution in which the concentration of hydrogen ion is 6×10^{-8} ?

9. What is the hydrogen ion concentration of a solution which has a pH = 7.5?

10. How does the titration curve of a weak acid differ from that of a strong acid when both are titrated with a strong base?

11. Sketch the titration curve of acetic acid titrated with sodium hydroxide and state in words how this indicates that a partially neutralized weak acid, i.e., a mixture of the acid and its salt, constitutes a buffer mixture.

12. What is the meaning of the two sharp points of inflection in the titration curve of orthophosphoric acid?

13. Why is it acceptable in writing the expression for the ionization constant of a weak acid in a buffer mixture to replace $[A^-]$ with $[\text{salt}]$ and $[HA]$ with $[\text{total acid}]$?

14. Write out the expression from which the $[H^+]$ can be calculated in a buffer mixture, and transform this into the expression for calculating the pH .

15. A given sample of blood contains 0.03 m μ l of $NaHCO_3$ and 0.0015 m μ l of H_2CO_3 . How will the pH change if to 100 ml. of blood, 90 mg. of lactic acid is added? pK_1 for H_2CO_3 is 6.36. (Hint: As in any buffer mixture the new acid will simply change the ratio of salt to acid. Calculate the added acid concentration in m μ l and consider what effect this will have on the concentrations of H_2CO_3 and $NaHCO_3$.)

16. If you wanted to make up a buffer that would have a pH of 6.5, how would you decide what weak acid to use?

17. What will be the hydrogen ion concentration of a solution if 3 ml. of 0.05M disodium hydrogen phosphate solution is added to 4.8 ml. of 0.05M sodium dihydrogen phosphate?

18. What is meant by the "colloidal state"? What are the two types of colloidal particles? How do they differ?

19. Why are colloids of one type especially sensitive to added electrolyte? Why is the other type precipitated by very strong solutions of ammonium sulfate?

20. What is meant by "reversal" of an emulsion? Indicate one way in which this may be brought about.

21. What is meant by a "Donnan equilibrium"? Under what conditions is such an equilibrium set up? Why is it of biological interest?

The Structure of Living Forms

All the physiological activities of animals and plants—assimilation, secretion, excretion, motion, generation—are the expression of the activities of the cells considered as physiological units.

THOMAS HENRY HUXLEY (1887)

The enormous variety of plant and animal forms would seem at first to present insuperable obstacles to any comprehensive study of their chemistry. Defining a species as "a group of individuals alike in essential features of form and structure," there have been described about 800,000 species of animals and nearly 400,000 species of plants. The problem of describing and classifying this confusing multiplicity of forms engaged the attention of zoölogists and botanists for hundreds of years from the days of Aristotle. Then in the course of about twenty years in the middle of the nineteenth century came two great generalizations which served to emphasize the fundamental qualities common to all living organisms, and thus to simplify a large and complex field of knowledge. These two were the Cell Theory and the Theory of Organic Evolution.

Nature and Activities of Living Cells

It is now generally accepted that the physical unit of living tissue above the lowest levels of organization is the cell, and that each cell arises from a previously existing one. This basic fact points up the close relationship that must exist between the most diverse living forms. Those now present on the earth are believed to have evolved from simpler forms, and these in turn from still more rudimentary organisms. The simplest forms revealed by fossils must be quite complex compared with those which, lacking any hard integument or skeleton, have left no record. At some time well over 350 million years ago life began, perhaps as a single cell, perhaps in a less organized form. From this beginning has arisen the whole complex variety of higher animals and modern vegetation. Numerous and different as are the present-day offspring of that dimly imagined primordial organism, they are yet alike in this, that every living cell on earth must acquire and degrade foodstuffs in order to obtain building material and energy to maintain itself. As a preliminary to an examination of this

process as it is carried out by higher organisms, it may be instructive first to see how it is achieved by a single cell.

CELL STRUCTURE

Microscopic examination of plant and animal tissue reveals the fact that while their cells differ markedly in function and shape and size, as long as they are living they have certain fundamental characteristics in common. The existence of a nucleus in plant cells was first noted in 1831 by the Scots botanist, Robert Brown, though he did not recognize its true importance. A few years later Felix Dujardin (1801–1860) described a semifluid, jelly-like substance in animal cells which he believed to be

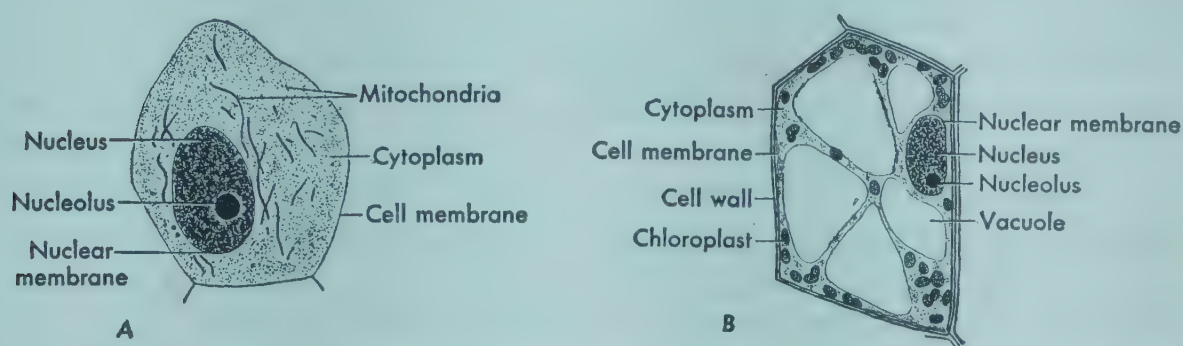


Figure 3.1. The essential features of a typical animal cell (A) and a typical plant cell (B).

endowed with all the qualities of life, and which he called *sarcode*. In 1846 a similar jelly-like material was found by Hugo von Mohl (1805–1872) in plant cells and was named *protoplasma*, meaning “first thing made.” By 1850 scientists had begun to wonder if the sarcode of the zoölogists were not identical with the protoplasm of the botanists and finally in 1861 Max Schultze (1825–1874) definitely made this generalization. Protoplasm is now recognized as the physical basis of life, the material which makes up the essential cell whether or not there is a visible cell wall. A unicellular plant or animal consists of a single, highly organized bit of protoplasm. Even as complex an organism as a man or a tree is made up entirely of protoplasm and the substances which protoplasm has synthesized in the course of development.

The appearance of typical plant and animal cells is shown diagrammatically in Figure 3.1, in which the protoplasm is seen to be far from homogeneous. Of the great number of specialized structures within the cell, the nucleus is most conspicuous and most important. It consists of an approximately spherical body which appears denser than the rest of the cell, and is bounded by a membrane. Its general importance is attested by the fact that if a unicellular organism is cut in half, only the portion which contains the nucleus will regenerate. It is found also that the nucleus serves a unique function in cell division, and that it is probably the physical agency through which hereditary traits are transmitted.

The remainder of the protoplasm outside the nucleus is spoken of as the *cytoplasm*. It appears granular because of the presence of a great number of minute structures which are scattered about in it. In plant cells these structures include the small bodies known as *plastids*, some of which contain chlorophyll and are called *chloroplasts*; others of the plastids may contain other pigments while some are colorless and are known as *leukoplasts*. In all cells there are numerous tiny granules known collectively as *chondriosomes* or *mitochondria*, some of which are spherical while others are threadlike. For many years the functions of the mitochondria were entirely unknown. But it has recently become possible to separate these particles by differential centrifugation from their cytoplasmic matrix and so to determine their specific contribution to the cells. It has now been shown that these tiny bodies possess all the necessary catalysts to bring about a wide variety of oxidative reactions and it may well be that the entire oxidative mechanism of the cell is localized in them.

Some animal cells have a structure known as the *centrosome* at one side of the nucleus, and may also carry localized about the centrosome or scattered through the cytoplasm the components known as *Golgi bodies*. When the centrosome is present it plays a part in cell division; the function of the Golgi bodies is not known. Besides these integral parts of the cell, there may be present food particles such as fat droplets or, in plant cells, the starch grains which serve them as food reserves.

The entire cell is enclosed in a fine semipermeable membrane which in animal cells may constitute their only boundary. Indeed in some tissues its presence has to be inferred from the way in which the cells react to solutions, since the membrane is too fine to be visible under the microscope. Such membranes are believed to consist of lipids and proteins arranged in a definite pattern to form large *lipoprotein* molecules. If vacuoles are present in the cell body there is a similar membrane between them and the cytoplasm, so that every substance which reaches the interior of the cell must pass at least one such membrane. In plant cells the delicate plasma membrane is supported by a cell wall which often contains large quantities of cellulose.

CHEMICAL CONSTITUTION OF PROTOPLASM

The early analytical studies of protoplasm must have been somewhat disappointing, for they uncovered no clue to its extraordinary properties. The elements which are present are in no sense unusual. The cell is made up largely of carbon, hydrogen, oxygen, and nitrogen, with smaller amounts of the metals potassium, sodium, magnesium, and calcium, and of the nonmetals sulfur, chlorine, and phosphorus. Other elements are present in traces, among them iron, copper, iodine, and manganese.

If protoplasm contains no very exotic or uniquely interesting elements, neither is it built of compounds which are in themselves particularly

remarkable. The greater part of the cell content is water which constitutes over 90 per cent of the total weight in many tissues, and probably accounts for at least 75 per cent of the weight of all living cells. Dissolved or suspended in the water are a number of organic and inorganic compounds. The metallic chlorides, sulfates, and phosphates, present largely in ionic form, make up only a small percentage of the total "dry weight." The greater part of the nonaqueous part of protoplasm consists of organic compounds such as proteins, fatty substances, and carbohydrates, with small amounts of other compounds, many of them containing nitrogen. The simpler carbohydrates, the mono- and disaccharides, are present in solution, but the larger molecules give to the cell contents its colloidal form, on which in turn depend many of the characteristic properties of protoplasm. It is probable that many of the ions and the simpler molecules occur in combination with or adsorbed on the colloidal material of the protoplasm, and that they are only released during isolation procedures.

LIFE IN A SINGLE CELL

The amoeba, found in quantity in muddy water, is a familiar example of a single-celled animal (Fig. 3.2). Besides the nucleus and granular cytoplasm, there are to be noted inside the cell a vacuole which contains food particles, and a clear contractile vacuole. It is possible by watching the amoeba under the microscope for a time to see food vacuoles form and disappear, and to see the use of the contractile vacuole. If a bit of food is introduced at the edge of the drop of water on the microscope slide, the amoeba advances toward it in a haphazard sort of way by pushing out extensions of its own body in what are called *pseudopodia*, and then flowing along to catch up with the extension. It ultimately flows around the food particle and engulfs it, enclosing a droplet of water at the same time. Food and water are quickly separated from the animal's body by being enclosed in one of the small vacuoles which are visible in the drawing. At first the outlines of the bits of food are perfectly clear, but slowly these become blurred and the particles appear to dissolve. If there is any part of the food which cannot be made soluble it is ultimately rejected after the amoeba has flowed along and so brought the refractory material close to its own outer membrane. At various times the contractile vacuole seems to disappear and then re-form, and it is believed to be acting at such times to excrete from the body of the cell some soluble waste. Thus the amoeba digests its

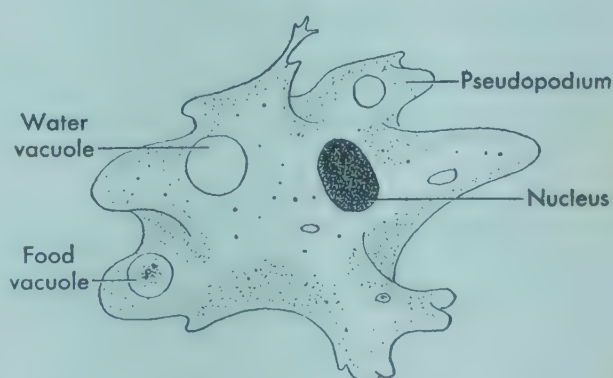


Figure 3.2. A unicellular organism, the amoeba.

food in an area temporarily set aside for the purpose and the digested fragments dialyze out into the cytoplasm.

If the microscope slide is marked off in squares so that the size of the amoeba can be measured from time to time, it will be seen to grow. Somehow in the subtle chemistry of its tiny laboratory, food is being transformed into protoplasm.

In addition to food the amoeba must have oxygen which it obtains from the air dissolved in the water in which it lives. Oxygen is used to oxidize part of its food, as evidenced by the continuous evolution of carbon dioxide. This oxidation provides energy for motion and for the synthetic reactions by which it transforms part of its digested food into protoplasm. In thus using molecular oxygen in a reaction which makes available to an organism the chemical energy locked up in organic molecules, the amoeba is typical of the vast majority of living cells.

Plants and Animals

Given the basic fact that living cells must be able to obtain energy and to build or maintain protoplasm, let us next look briefly at the ways in which this is accomplished in plants and in animals.

HABITS AND STRUCTURE

Most higher plants are green; most plants are fixed in position while most animals are motile; many plants are heavily branched, with hundreds

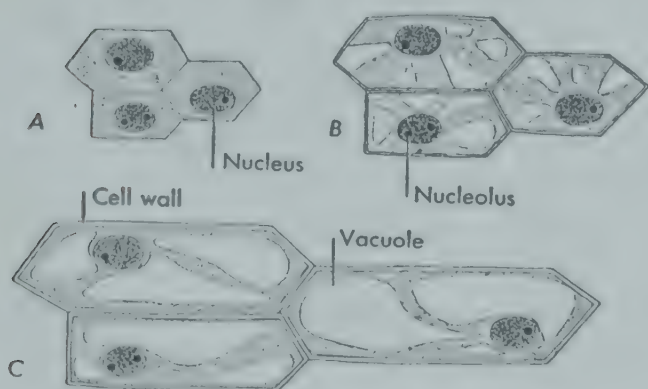


Figure 3.3. Plant cells at three different ages from the very young cells (A), in which the cell walls are filled with protoplasm, to mature cells (C) in which the cell contents have become little more than a lining of the cell walls.

of identical or similar parts, while animals are more compact and less repetitive structurally.

Closer examination reveals other characteristic differences of habit and structure. An animal grows all over its body, so that at one time its internal organs, its limbs, its brain, and its skeleton are all increasing in size. This continues until it reaches adulthood when growth normally stops. But although the cells cease to grow and

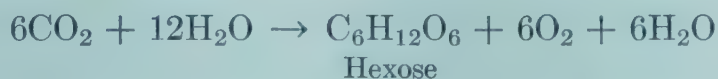
multiply most of them remain actively alive. In contrast to this, plant growth is largely localized, but goes on continuously so long as the plant lives. The tips of the root and the tips of stems grow. The part of the plant behind these growing areas gradually takes on a different structure and becomes a rigid skeleton and a passageway consisting largely of dead cells.

A thin and often invisible surrounding membrane is the only barrier separating one animal cell from another, and normally that membrane is completely filled with protoplasm. Plant cells on the other hand have thick and conspicuous cell walls which help to support the plant. Inside this wall is the semipermeable plasma membrane which in young cells is almost filled with protoplasm. But as plant cells grow older and are left behind the growing tip, they elongate and their protoplasmic contents are transformed into a thin lining within the rigid cell wall (Fig. 3.3). This leaves a large central space or vacuole which holds a watery solution known as cell sap.

USE OF SOLAR ENERGY

Of the differences enumerated above between plants and animals, the first is by all odds the most fundamental. The possession of the pigment chlorophyll makes possible the *autotrophic* or self-nourishing metabolism characteristic of green plants.

Plant cells like those of animals must oxidize organic molecules to obtain energy. But they differ from animal cells in having the ability to build these up by photosynthesis, that is, to synthesize essential food-stuffs from simple inorganic molecules at the expense of energy derived from sunlight. In the presence of chlorophyll and of sunlight, plant cells reduce carbon dioxide to carbohydrates through a complex series of reactions which will be considered later. Meantime the skeleton reaction is now formulated



in conformity with the experimental evidence that all of the oxygen evolved in photosynthesis comes from water molecules. In the simple sugar thus formed, solar energy is stored as chemical energy which may later be released and used by the plant when the compound is oxidized.

But photosynthesis has an importance which far transcends its utility to the plant itself. Animals, lacking chlorophyll, are unable to use radiant energy. They are therefore dependent upon a supply of organic foodstuffs in which the chemical energy lies ready to their use. This supply they obtain either by eating plants or by eating other animals. Thus the organic compounds of the animal body are derived either directly or indirectly from plant products, and in the final analysis it is the green plants which make available to nearly all the living organisms on the earth the great resources of solar energy.

ALTERNATIVE ENERGY SOURCES

Most organisms follow the pattern which has just been sketched and derive their energy directly or indirectly from solar radiation. But among

the microorganisms there is interesting evidence of the great versatility Nature has shown in maintaining life on the earth. Most bacteria are *heterotrophs*, that is, they are like the animals in being dependent upon some outside source of organic food. Other microorganisms such as the green algae and a few bacteria contain chlorophyll, and these are autotrophic as are all other organisms which have been provided with this useful catalyst. But there are a few types of bacteria which, although they lack chlorophyll, still are able to live independently of a source of organic foodstuffs provided certain inorganic substances are available. These organisms obtain the energy they need not from sunlight but from exothermic chemical reactions. The energy thus made available is used as green plants use solar energy to bring about synthesis of foodstuffs from carbon dioxide. Among the reactions which are thus coupled with reduction of carbon dioxide to carbohydrates are the oxidation of ammonia to nitrite and of nitrite to nitrate, as well as oxidation of sulfur, of hydrogen sulfide, and of ferrous compounds.

One imagines that in the course of evolution various possible sources of energy for living organisms have been explored. The widespread development of green plants proves the success of the method in which chlorophyll takes part. The varied forms of animal life indicate that the other really efficient method has been that in which heterotrophic organisms have diverted to their own ends a share of the energy stored up by plants. That oxidation of small inorganic molecules has not been highly esteemed by Nature may be deduced from the fact that it is used by only a few unicellular organisms. It is evidently not a plan which can be adapted to the needs of larger and more complex forms. This is easy to understand in the case of a metabolism which requires that an organism live in an atmosphere of hydrogen sulfide, and deposit granules of sulfur within its cell walls!

Multicellular Organisms

As with the amoeba, the metabolism of all unicellular organisms whether plant or animal depends upon direct contact with their aqueous or moist environment. They obtain from this everything that they need either by diffusion or by engulfing small food particles. With equal directness waste materials are expelled or diffuse away. Even some small multicellular organisms such as the hydra have managed to remain in intimate contact with their aqueous medium by developing a crude, infolded digestive tube (Fig. 3.4). Such an organism is not more than two cells thick, every cell is bathed by water, external or internal, and diffusion takes care of all its needs.

As organisms became larger and migrated to dry land they had to develop indirect ways of keeping their cells alive. It was necessary to

get water into interior cells, and at the same time to prevent its entering in such volume as to burst the cell walls. Cells far from the surface had to be supplied with food and oxygen, and to be relieved of waste matter. Plants and animals have solved these problems by the development of different organs, but both have had recourse to some sort of division of labor. This has required a complex organization and structure, with each part of the organism carefully integrated with every other part.

PLANT STRUCTURES

Plants are in general simpler in organization than animals, needing only three fundamental structures—the root, the stem, and the leaf—to carry on the life of any individual plant. In the course of that life flowers and fruit may form or other reproductive structures may arise, but these are not essential to the life of the parent plant.

Roots and Stems. In such a representative plant as a tree its supply of water and of inorganic compounds is taken in by the microscopic root hairs which are in close contact with the soil. Thence the watery solution moves upward through a series of fine tubelike cells in the centers of roots and stems, which carry it finally along the branches and into the leaves. The tissue concerned with this upward transportation of material is known as *xylem* or wood. It is made up of long slender empty cells with firm supporting walls. These cells connect with each other through thin places in their walls, and are so arranged that there is a continuous conducting system from the roots to every branch and leaf. Part of the water which rises into the leaves is continuously being lost in *transpiration* or evaporation. This loss is minimized by the presence of the impervious waxy cuticle which covers most of the leaf surface, and of the corklike bark, made up of dead cells, which covers the stems.

In addition to the xylem system which carries material up from the soil, there is a second series of tubelike cells involved in the transport of material in the plant. It lies parallel to the xylem and serves to carry downward to all living parts of the plant the photosynthetic products which constitute the food supply for the whole organism. The tissue involved in downward transport is known as *phloem*, the chief cells of which have perforated end walls to facilitate the passage of fluid. Figure 3.5 shows two typical arrangements of phloem and xylem tissues. In

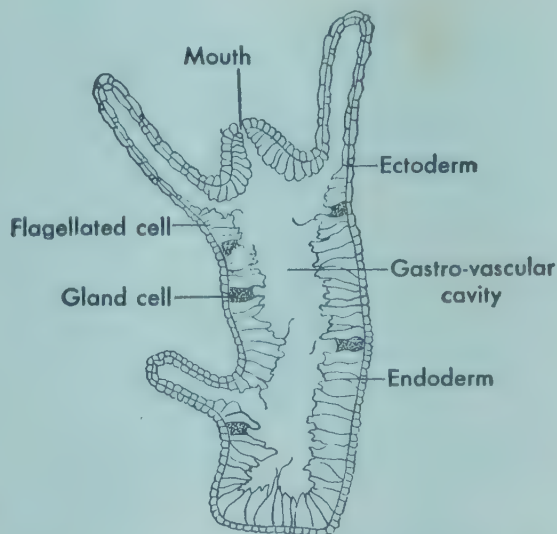


Figure 3.4. A multicellular organism, the hydra, the cells of which are all in contact with its aqueous environment.

(a) small bundles made up of both types of vascular tissue form a circle around the periphery of the stem; in (b) the xylem forms a cylinder inside the phloem layer.

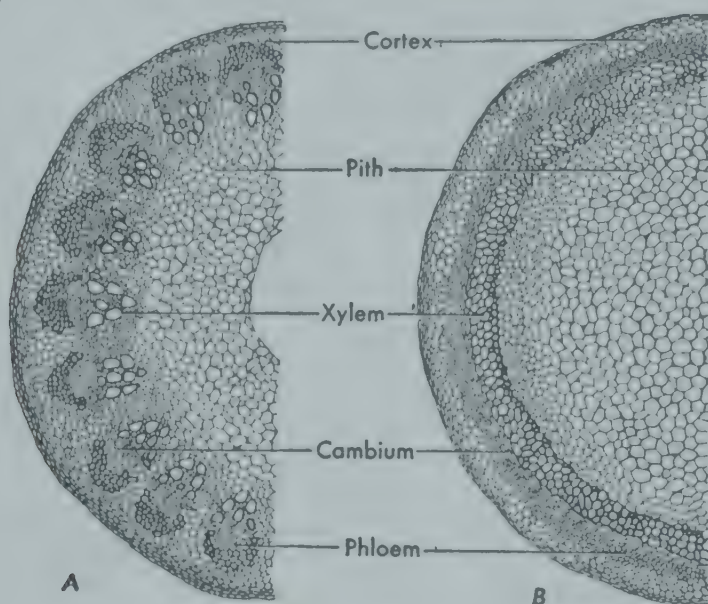


Figure 3.5. Cross sections of two stems to show the disposition of xylem and phloem.

Leaves. The bulk of the living part of a plant consists of leaves, and the structure of these is admirably adapted to giving them access to carbon dioxide and to facilitating the disposal of the oxygen set free in photosynthesis. Leaves are very thin and have the porous structure shown in Figure 3.6. A waxy cuticle, in conjunction with an upper and a lower epidermis which are usually only one cell in thickness, serves to minimize



Figure 3.6. The essential structures of a leaf, showing how all the cells have free access to air. Note also the vascular bundle of xylem and phloem tissue making up the leaf vein.

the loss of water. The inner cells make up the *mesophyll*, and all contain more or less chlorophyll. In the center of the figure are shown the vascular cells of the leaf vein, through which watery solutions are transported from and to the leaf. The small openings in the lower epidermis, the *stomata*, are the paths by which all gas exchange in the leaf takes place. The water which is lost in transpiration passes out through the stomata; carbon dioxide diffuses inward and thus becomes available to the chloro-

plants. As photosynthesis proceeds, oxygen is set free and part of this in turn escapes through the stomata during daylight hours. In the dark the plant is entirely dependent upon oxygen of the air which diffuses in to serve its respiratory needs.

To summarize: Even the most complex plants get their raw materials from the soil in the form of an aqueous solution and from the air as carbon dioxide. Because the stomata open into a porous ramifying structure only a few cells in thickness, leaf cells are all in direct contact with the gases which diffuse in. Since plants synthesize their own primary foodstuffs they have no need of a digestive system, though something resembling digestion must take place whenever a cell begins to use its stored foods. If a plant excretes any waste material except oxygen the amount is small compared with its total metabolism. Some crystals occasionally found in plant cells are insoluble calcium salts of acids which are thought to be waste products. Lacking a means of extruding these acids the plant cell simply precipitates them within its own walls.¹

The essential organs of a higher plant are: (1) widely ramifying roots, from the surfaces of which project fine root hairs, each being a thin-walled projection from a single cell and so very effectively in contact with soil moisture and air; (2) stems with few nutritive needs by way of which solutions pass upward from the roots and downward from the leaves; and (3) an enormous number of flat, thin leaves perfectly adapted to absorbing sunlight and to allowing passage of carbon dioxide and oxygen through the stomata.

ANIMAL STRUCTURES

The evolution which has set animals free to wander anywhere on the surface of the earth has also moved them farther than most plants have gone from effective contact with an aqueous environment. The problem thus posed is the more serious in that life is possible only within a rather narrow range of conditions. Salt concentration, osmotic pressure, and acidity can vary only slightly from optimum conditions if cells are to survive. To ensure the required uniformity of what Claude Bernard called the "internal environment" of their cells, animals are equipped with a more complex group of organs than plants have developed. In noting the essential systems in the following paragraphs, the reproductive organs are omitted since they play no large role in the metabolism of the parent organism.

¹ The animal kingdom uses a similar mechanism with embryos which develop inside an impervious shell. The usual nitrogenous waste of animal cells is the water-soluble amide, urea, which is toxic if present in appreciable concentrations. If this were formed by bird and reptile embryos it would soon reach a lethal concentration in the shell. Instead these species excrete waste nitrogen as insoluble uric acid which collects as crystals inside the shell and is thus effectively out of contact with the living tissues.

The Circulatory System. In most higher animals the transportation of food and oxygen to the cells and the removal of waste from the cells is entrusted chiefly to a system of vessels through which blood of some kind is driven by a heart of greater or less complexity. The effectiveness of such a system depends upon its having places of intimate contact with air and with sources of food as well as with the tissues which must be nourished. This contact between blood and tissues is achieved by a continuous branching of the *arteries* leading from the heart so that they form smaller and smaller vessels which finally become tiny capillaries with walls only a single cell in thickness. These thread their way through a section of tissue and then gradually coalesce again to form the first small *veins* through which the blood begins its journey back to the heart. In the capillary beds, exchange of material between blood stream and tissue cells is a simple matter of diffusion regulated by a concentration gradient. Thus the blood acquires foodstuffs in the digestive tract and oxygen in the respiratory organ and distributes them to all parts of the organism.

Aside from its function as a common carrier, the chief contribution made by the blood itself to the animal economy is the *respiratory pigment* by virtue of which a given volume of blood carries far more oxygen than it could hold in simple solution. Among vertebrates this pigment is *hemoglobin*. A similar substance, *hemocyanin*, which is found in the blood of molluscs and crustaceans, is blue in oxidized form and colorless when reduced, while certain worms produce a green pigment known as *chlorocruorin*. Whatever the exact nature of the pigment it acts by virtue of its ability to form a loose compound with oxygen whenever the oxygen pressure is high, and to release the gas when the oxygen tension falls. Thus as blood flows through the capillaries of the lungs or gills the pigment goes over into the oxygenated form; later when this blood flows through capillaries deep in the tissues it comes into close contact with the extracellular fluid in which all the body cells are bathed. Here the oxygen pressure is comparatively low and the pressure of carbon dioxide is high. Oxygen dialyzes out into the fluid, and thence across cell membranes into the cells. Meantime carbon dioxide passes into the blood, as do other waste products such as urea and uric acid. The carbon dioxide is carried to the lungs or other respiratory tissue for excretion; the soluble wastes are dealt with by the kidney or some similar organ.

The Lymphatic System: In addition to the rapidly circulating blood stream there is another fluid which moves sluggishly through the *lymphatic system*. The extracellular fluid referred to above is essentially a blood filtrate which is driven through the capillary walls by the blood pressure. In composition it is very like the blood plasma, except that its protein concentration is relatively low. To remove this fluid continually from the tissue spaces into which it is pressing, the mammalian body is provided with a series of drainage channels or lymph vessels. These begin as blind

tubes into which the fluid is forced by its own growing pressure in the tissue spaces. These vessels converge upon each other, forming larger vessels which lead ultimately into two main ducts, the *lymphatic* and *thoracic* ducts. These empty their contents into the veins near the heart, thus bringing back to the blood the fluid which filtered out in the tissues. Here and there the larger lymphatics are interrupted by *lymph nodes* whose chief function seems to be filtration of microorganisms. Since this system has no heart the fluid moves slowly, driven along on the one hand by the pressure of the fluid coming into the tissue spaces, and on the other by the contraction of muscles along the lymphatic route.

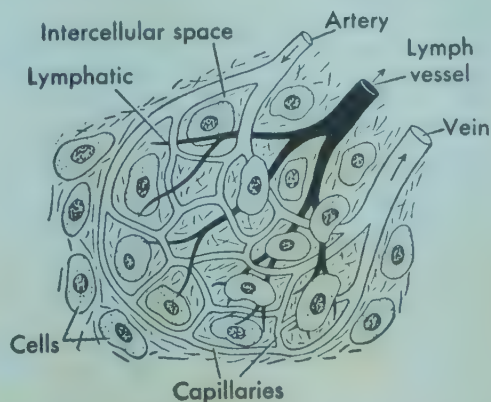


Figure 3.7. Diagram of the relation between tissue cells and blood and lymph capillaries. Note that the blood capillaries form passageways from the artery to the vein, while the lymph vessel begins as blind tubes. (Reprinted with permission from W. C. Curtis and M. J. Guthrie, *General Zoölogy*, 1947, John Wiley & Sons, Inc.)

It may at first sight seem strange that any molecules as large as those of proteins are able to migrate from the capillaries into the tissue spaces. It may be that some of them pass through spaces between cells. But it must also be remembered that the membranes which we see under the microscope, and which are drawn as continuous lines, consist of large, unsymmetrical molecules. These are chiefly proteins and fatty substances piled into a sort of dry wall which will inevitably include many open spaces. Through such spaces protein molecules might well find their way.

In Figure 3.7 is shown the relation between cells, extracellular fluid, lymph vessels, and capillaries. It is clear that the individual cell of a multicellular organism is in the position of a very fortunate amoeba. Its food supply is not a matter of chance encounter, but is delivered in pre-digested form by capillary. Its waste products are carried away and it is free to get on with whatever work it has to do. Together, the blood and lymph provide it with that stable internal environment of which Claude Bernard spoke.

Respiratory Organs. In the lower reaches of the evolutionary scale, respiratory organs are not needed, even by multicellular forms. In earthworms and amphibia, for example, a large part of the respiratory exchange takes place through the thin, moist skin. But in order that the blood of larger forms may acquire a sufficient supply of oxygen, various more or less effective respiratory organs have been developed. Aquatic forms depend for their oxygen upon the small amount in aqueous solution, and this

makes it necessary to bring the blood into contact with large volumes of water. This is achieved by passing it through gills, the thin sheets of which are highly vascular. A continuous stream of water bathes the gill tissue, and the great number of fine capillaries facilitates the passage of oxygen from this water into the blood.

Those animals which have moved into a terrestrial environment have the advantage over aquatic animals that their source of oxygen is the much more concentrated air. On the other hand, in order that diffusion may take place their lungs have to be kept moist, and the rather heavy fluid layer which coats the lining of the lungs to prevent their drying, also greatly reduces the possible speed of diffusion. To offset this slow passage, the area of the lung epithelium is greatly enlarged by the presence of a vast number of tiny branched sacs, the *alveoli*, which give to that tissue its spongy appearance. It is estimated that the internal respiratory area of adult human lungs is about 100 square yards. Here as in the gills, the tissue is very vascular, so that all of this large area is in close contact with capillaries.

The type of oxygenation developed among the insects is of interest because it provides another example of a system which has been efficient enough to survive, but has not proved adaptable to the needs of larger forms. The air required by insects is taken directly to all the cells by means of many pairs of fine, branching tubes, or *tracheae* which are open to the air. Some of these end blindly in the deepest tissues while others communicate with the tubes on the opposite side, and thus establish an air passage through the animal. Air is kept moving by contraction and expansion of the body walls; it gives up its oxygen directly to the cells and carries excretory carbon dioxide with it as it moves out to the surface again. With such contact between air and even the deepest tissues, the insect needs only a most rudimentary circulatory system, in spite of the fact that its muscular activity and therefore its energy requirement is relatively large. But although this arrangement is quite satisfactory for organisms the size of a grasshopper, it is not found in larger forms. In the evolutionary struggle the special vascular gill or lung tissue, coupled with a rapid circulation of blood, seems to have proved better adapted to supplying the oxygen needs of organisms of greater size.

The Digestive System.² In multicellular animals the digestion of food takes place in organs specifically adapted to perform this function. The simplest forms in which this division of labor becomes apparent are those previously mentioned which have formed a short digestive tube by folding one layer of cells within another. Digestive juices are formed by the cells which line this primitive gut and are poured over the food there. As the food is digested the fragments diffuse directly into all the cells. It is probable

² This system is dealt with at greater length in Chapter 10.

that in such forms as these some intracellular digestion goes on as well and that all the cells are able to elaborate digestive catalysts. As forms evolved which were higher in the evolutionary scale digestion became confined entirely to a digestive tube and the cells outside the digestive area lost their ability to engulf and digest their own food particles. Finally in the mammals the gut became longer in relation to the length of the animal and was enlarged locally to form one or more stomachs where food could be held to give time for the digestive juices to act. Since herbivorous animals must consume large volumes of food, their intestines have come to be even longer than those of carnivores. In cats, for example, the alimentary canal is three to five times as long as the body, but cows and horses have intestines over twenty times their own length.

With increasing complexity and size came also further subdivision of labor. From certain specialized cells set aside to perform a specific secretory function there developed quite early in evolutionary history such secretory organs or *glands* as the liver and the pancreas. One function of these glands is the elaboration of some of the digestive catalysts. Even among mammals, however, part of the secretory activity is still carried out by cells which make up part of the lining of the digestive tract. The gastric juice and the hydrochloric acid which together initiate digestion in the stomach are each secreted by specialized cells in the gastric mucosa, while secreting cells in the lining of the upper intestine provide the intestinal juice. The relation to each other of the various digestive organs in man is shown diagrammatically in Figure 3.8.

Excretory System. Animals have two kinds of waste material which must be eliminated. There is the solid or semisolid matter which passes to the end of the digestive tract and is there excreted as feces. But in the course of metabolism there are also formed soluble wastes which must be removed. In some of the lower forms this is achieved by means of a system of single tubes or *nephridia* which lead from the tissues directly to the exterior.

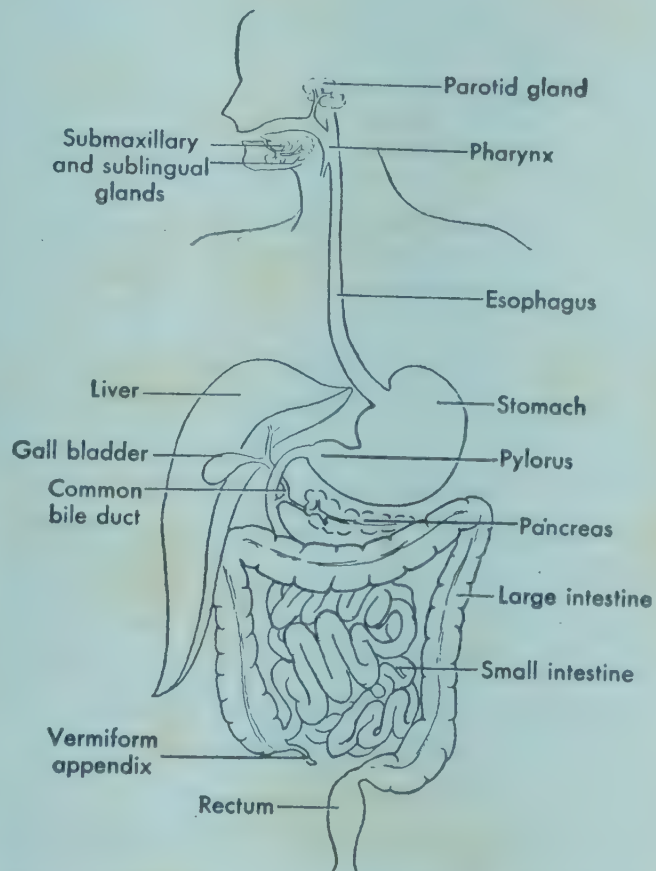


Figure 3.8. Diagrammatic representation of the digestive tract in man.

Cilia located near the internal ends of the tubes set up vibrations which draw fluid in and then propel it to the outside (Fig. 3.9).

Clearly related to these primitive forms are the *uriniferous tubules* which make up the kidney in higher animals. They do not open directly into

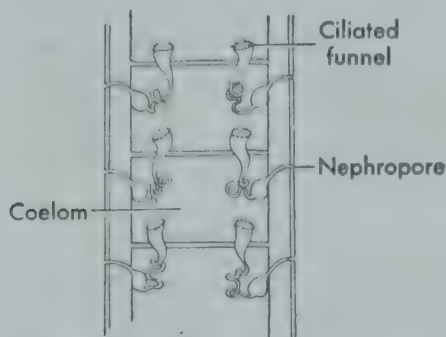


Figure 3.9. A simple excretory system, the paired nephridia of the segmented worms.

the body cavity as the nephridia do, but each one begins near the outer surface of the kidney as a knot of arterial blood vessels enclosed in a funnel-like structure known as Bowman's capsule. As the blood flows through this glomerular knot, water and substances in solution are filtered from it and pass into the long convoluted tubule. The circulation of the kidney is so arranged that blood which has passed through Bowman's capsule flows next into vessels which send a network of capillaries around

the very tubule through which the blood filtrate is moving. During this passage certain substances including large amounts of water are reabsorbed; at the same time other constituents of the urine are secreted into the tubules along these lower walls. By the process of reabsorption the organism is enabled to recover water and other valuable substances which have passed into the glomerular filtrate. Whatever is not reabsorbed passes into collecting tubes which lead eventually to the bladder in mammals, or into the terminal part of the alimentary canal in lower vertebrates and birds.

Endocrine Glands. We have already seen that in higher animals part of the digestive juices are elaborated in two special secreting organs, the liver and the pancreas. These secretions flow into the upper intestine by way of the common bile duct and there act upon the food mass. Such fluids as these, formed in a gland and acting in a specific locality, are known as *external secretions*. But in addition

to its digestive juice the pancreas also secretes from specialized cells known as "islet cells" a totally different kind of substance which has nothing to do with digestion of food. This is the protein insulin which is absorbed directly from the pancreatic tissue into the blood stream. It

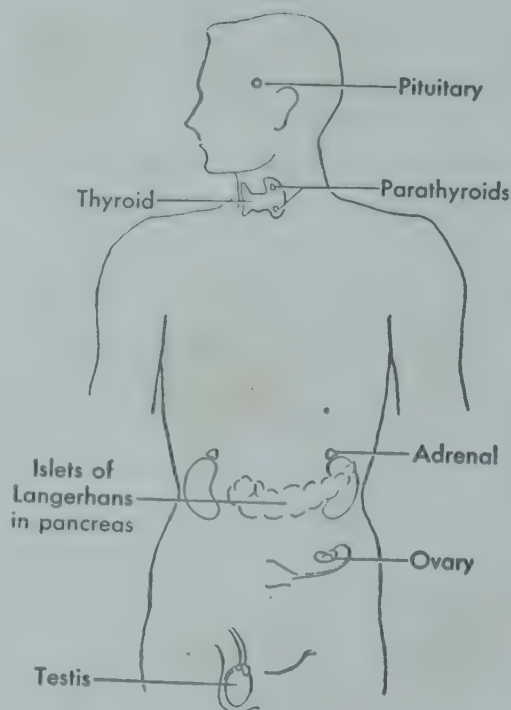


Figure 3.10. The location of the endocrine glands in man.

is thus carried to all parts of the body and acts upon various tissues far removed from its source. Such a substance is known as an *internal secretion* or *hormone*. Insulin is one of a number of such chemical messengers, secreted by different glands. Figure 3.10 shows the location in man of the chief glands of internal secretion, known also as *endocrine glands*. Table 3-I lists these glands with their principal hormones. The

TABLE 3-I. THE MAJOR ENDOCRINE GLANDS IN MAMMALS

Gland	Hormone	Principal Known Function
Pituitary		
Anterior lobe	Gonadotrophins	Stimulation of gonads
	Lactogenic hormone	Stimulation of milk secretion
	Adrenotrophin (ACTH)	Stimulation of adrenal cortex
	Thyrotrophin	Stimulation of thyroid
	Growth hormone	Growth of bone and muscle
Posterior lobe	Oxytocin	Contraction of smooth muscle
	Antidiuretic hormone	Control of water metabolism
Ovary	Estradiol	Control of the sex cycle, of pregnancy and lactation
	Progesterone	
Testis	Testosterone	Development of accessory sex organs
Adrenal cortex	Cortical steroids	Metabolism of electrolytes and of carbohydrates
	Epinephrin (adrenaline)	Control of circulation
		Control of deposition of glycogen
Thyroid	Thyroxin	Control of metabolic rate
Pancreas (islet tissue)	Insulin	Control of several phases of carbohydrate metabolism
Parathyroid	Parathormone	Metabolism of calcium and phosphorus

commanding position of the pituitary gland is especially noteworthy since it is upon the normal secretory activity of this one small organ that the functioning of most of the other glands depends.

The Aims of Biochemistry

The chemical problems set by living tissue are of course greatly simplified by the fundamental resemblances between individual cells, whether plant or animal. In order to elucidate the nature of the chemical transformations by which living cells transmute food and oxygen into energy and protoplasm, biochemistry has pursued more or less simultaneously two separate but related aims. In its early years it sought almost exclusively the complete chemical analysis of cellular material. This phase of the investigation is now complete in its major outlines. In the last years of the nineteenth century, and the early years of the twentieth, the spectacular progress of organic chemistry, united with the analytical genius of such men as Otto Folin at Harvard and Stanley Benedict at Cornell,

provided a knowledge of the chemical and physical properties of the major cell constituents.

The second, more truly biochemical aim of this borderline science was first served by a small group whose primary training was in medicine and physiology. Viewing the cell always as an integrated, living unit they yet believed that its chemical events could be made clear. It is hard to realize now, at the mid-century, that no more than twenty or thirty years ago some biologists were still insistent that comparison of the living cell with any other physicochemical system was sheer impertinence. They believed that protoplasm had special properties of its own, entirely independent of the actual molecules of which it was constructed, and that even if we knew down to the last atom every single molecule and ion within a cell wall, we could not hope to explain its activity in terms of the ordinary laws of chemistry and physics. One of the earliest champions of the contrary view was F. G. Hopkins of Cambridge University who said of the cell in 1913: "Its life is the expression of a particular dynamic equilibrium which obtains in a polyphasic system." Twenty years later he defined the "essential or ultimate aim" of biochemistry as "an adequate and acceptable description of molecular dynamics in living cells and tissues." Today we accept without question the belief implicit in that statement, that molecular dynamics in the cell follows an orderly pattern, which is the result of the functioning of the same chemical and physical laws which govern matter outside a cell wall. In the following chapters we shall see both how far biochemistry has progressed toward that "adequate description," and also how many problems remain unsolved.

Suggestions for Further Reading

Since this chapter gives only a brief review of biological material which is pertinent to a study of cell metabolism, almost any good textbook of Botany or Zoölogy would be enlightening. Especially recommended for general interest are:

GERARD, R. W., *Unresting Cells*, Harper, New York, 1940.

HOGBEN, L., *Principles of Animal Biology*, Allen and Unwin, London, 1940.

LOCY, W. A., *Biology and Its Makers*, Holt, New York, 1908.

MILNE, L. J., and MILNE, M. J., *The Biotic World and Man*, Prentice-Hall, New York, 1952.

Punnett, R. C., "Forty Years of Evolution Theory" in *Background of Modern Science*, edited by NEEDHAM and PAGEL, Macmillan, 1938.

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Part II

The Organic Constituents of Cells

The Chemistry of the Carbohydrates

For the study of the chemical processes in animals and plants there is, except for the proteins, no group of carbon compounds so important as the carbohydrates. . . .

EMIL FISCHER (1890)

The earliest sweetening agent was probably honey, but cane sugar came into use very early in Persia and Asia Minor. The culture of the sugar cane spread gradually westward across north Africa into Sicily and Spain, and thence, many years later, to the warmer parts of the western hemisphere. The need for some other source of sugar which would withstand the climate of northwestern Europe led to a wide search, especially in Germany. The plant which proved to be most suitable was the sugar beet which now furnishes most of the sucrose which originates in temperate lands. Meantime in the course of this search Marggraf had discovered in 1747 what he described as "a kind of sugar," present in raisins. This later proved to be the same substance which had previously been described in early Moorish writings. It was found to be present also in honey and in the syrup obtained by acid hydrolysis of starch, as well as in the urine of diabetics. It has been variously called dextrose, grape sugar, and glucose, but the latter name has now come into general scientific use.

The importance of the carbohydrates, to which class of compounds both cane sugar and glucose belong, is not limited to their use as sweetening agents. Green plants synthesize not only glucose but the important polysaccharides, starch and cellulose. The former is a reserve food of plants while the latter is present in the woody framework. These three substances, together with certain related compounds derived from them, have from remote times constituted the world's great storehouse of energy. In the form of oil and coal and peat the carbohydrates of the prehistoric past now serve as fuel for the mechanized present, while the carbohydrates elaborated by the plants of the present constitute one of the three primary foodstuffs. To these uses must be added also the importance of cellulose as raw material for the manufacture of a wide variety of materials including paper, explosives, artificial silks, lacquers, and paints.

The word *carbohydrate* reflects the early belief that carbon compounds which contain hydrogen and oxygen in the ratio 2:1 must be hydrates.

All of the most common and earliest known members of the group, sucrose, $C_{12}H_{22}O_{11}$, starch and cellulose, $(C_6H_{10}O_5)_x$, and glucose, $C_6H_{12}O_6$, can be represented by the general formula $C_x(H_2O)_y$, but it has long been recognized that other substances which clearly belong in the same chemical group are not even formally hydrates. Such for example are rhamnose, $C_6H_{12}O_5$, certain acids closely related to the simple sugars, and such nitrogen-containing compounds as glucosamine, $C_6H_{11}O_5(NH_2)$. Nevertheless the name is retained in modern parlance and is used now to designate

TABLE 4-I. PARTIAL CLASSIFICATION OF THE CARBOHYDRATES

Monosaccharides

TRIOSES $C_3H_6O_3$			
Glyceraldehyde	CHO—CHOH—CH ₂ OH		aldotriose
Dihydroxyacetone	CH ₂ OH—CO—CH ₂ OH		ketotriose
TETROSES $C_4H_8O_4$			
PENTOSES $C_5H_{10}O_5$			
Ribose, Arabinose, Xylose, and Lyxose			aldopentoses
Ribulose, Xylulose			ketopentoses
HEXOSESES $C_6H_{12}O_6$			
Naturally occurring aldohexoses: Glucose, Galactose, Mannose			
Naturally occurring ketohexoses: Fructose, Sorbose, Tagatose			
HEPTOSESES			
Mannoheptulose, Sedoheptulose			ketoheptoses

Oligosaccharides

DISACCHARIDES	
Sucrose:	yields on hydrolysis glucose and fructose
Lactose:	yields on hydrolysis glucose and galactose
Maltose:	yields on hydrolysis glucose
TRISACCHARIDES	
Raffinose:	yields on hydrolysis galactose, glucose, and fructose

Polysaccharides

HOMOPOLYSACCHARIDES: Compounds which yield on hydrolysis but one type of monose	
Dextrans:	yield glucose (dextrose) on hydrolysis
Starch:	reserve plant polysaccharide
Cellulose:	structural plant product
Glycogen:	reserve animal polysaccharide
Levans:	yield fructose (levulose) on hydrolysis
Inulin:	found in dahlia tubers
Polymers of other monoses or monose derivatives	
Xylans:	polymers of xylose
Chitin:	polymer of glucosamine
HETEROPOLYSACCHARIDES: Compounds which yield on hydrolysis more than one type of monose or monose derivative. Many of these compounds are still imperfectly characterized, and some of them yield also some noncarbohydrate units such as sulfuric acid or methyl alcohol.	
Gum Arabic:	yields on hydrolysis arabinose, rhamnose, galactose, and glucuronic acid
Hemicelluloses:	yield on hydrolysis a uronic acid and a pentose and may yield also methyl alcohol

nate the large group of organic compounds which either are themselves polyhydroxy aldehydes or ketones or closely related compounds, or yield such compounds on acid hydrolysis.

The carbohydrates are at present classified in three main groups. Simple sugars like glucose or fructose are known as *monosaccharides* or *monoses*; carbohydrates such as sucrose and raffinose which yield two or more, but fewer than ten, monosaccharide molecules on acid hydrolysis are called *oligosaccharides*; those which, like starch or cellulose, set free a large number of simple sugar molecules constitute the group of *polysaccharides*. The number of simple sugars in an oligosaccharide is indicated by such names as *disaccharide* or *tetrasaccharide*. In Table 4-I is given a classification of the more abundant and better-known members of the carbohydrate group.

Individual names were given to many of the sugars long before there was any rational system of scientific nomenclature. Thus glucose gets its name from the Greek word meaning sweet; fructose comes from the Latin *fructus* and indicates that this sugar occurs in many fruits, while the name of arabinose is a sign of its source in gum arabic. Modern classifications within the monosaccharide group make it possible to indicate in a class name something of the structure of these compounds. Glucose is an *aldohexose*, that is, a carbohydrate, indicated by the *-ose* ending, containing six carbon atoms, one of which is present in an aldehyde group. Similarly fructose, which has a carbonyl group at the second carbon, is a *ketohexose*, and arabinose with a chain of five carbons is an *aldopentose*.

The Monosaccharides

The important monoses are the trioses, the pentoses, and the hexoses. They are colorless crystalline solids having more or less sweet tastes. They are all soluble in water and form solutions which are optically active and which reduce Fehling's solution and ammoniacal silver nitrate.

Among the simple sugars glucose has a unique importance both in nature and in chemical history. It is a fundamental product of photosynthesis and is also the form into which most carbohydrates are converted before being used by living cells. Historically glucose was the first of the monosaccharides to be characterized and the one on which in 1884 Emil Fischer¹ began his classical study of the carbohydrates and their interrelationships.

¹ Even in that galaxy of great German scientists whose work made of the nineteenth century a golden age of organic chemistry, Emil Fischer (1852–1919) stands pre-eminent. His work on the constitution and stereochemistry of the sugars laid the foundation on which modern carbohydrate chemistry is built. But this was only one of his interests and his studies of the amino acids and the proteins opened up that whole fruitful field of research. It is suitable that the second Nobel Prize in Chemistry should have been awarded to him in 1902, the first having gone to van't Hoff in the previous year.

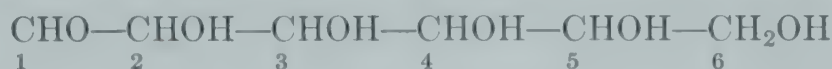
It is therefore appropriate to center our study of the monosaccharides around the chemistry of glucose.

THE ALDEHYDE REACTIONS OF GLUCOSE

The so-called "old structure" for glucose ($C_6H_{12}O_6$) had been formulated between 1868 and 1870, before Fischer's work began. It represents the sugar as a straight chain pentahydroxy aldehyde and rests upon the following reactions:

1. On reduction with sodium amalgam a hexahydroxy alcohol, sorbitol, is formed, which can in turn be further reduced by hydriodic acid to yield 2-iodohexane. This proves that the carbon chain in glucose is not branched.
2. On treatment with an acetylating agent such as acetyl chloride or acetic anhydride, a compound is formed which has the formula $C_{16}H_{22}O_{11}$. This represents the introduction of five acetyl groups, and points to the presence of five hydroxyl groups in the sugar molecule.
3. On gentle oxidation with bromine water, an acid, $C_6H_{12}O_7$, is formed. Formation of a carboxyl group by addition of a single oxygen atom indicates that the original molecule must have contained an aldehyde group.

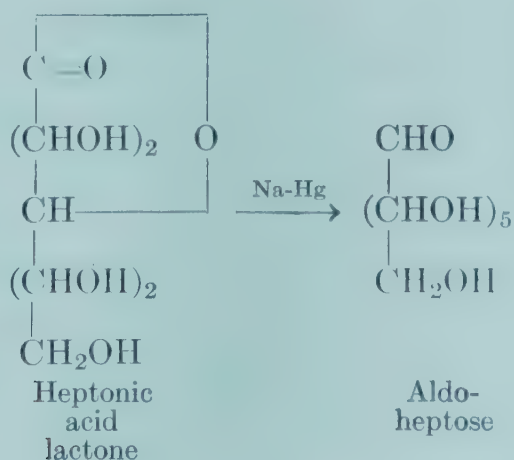
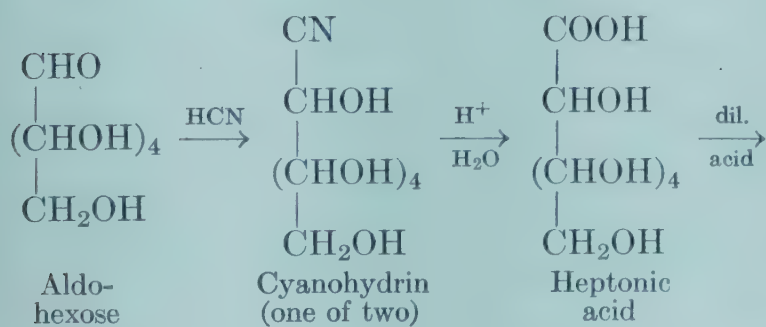
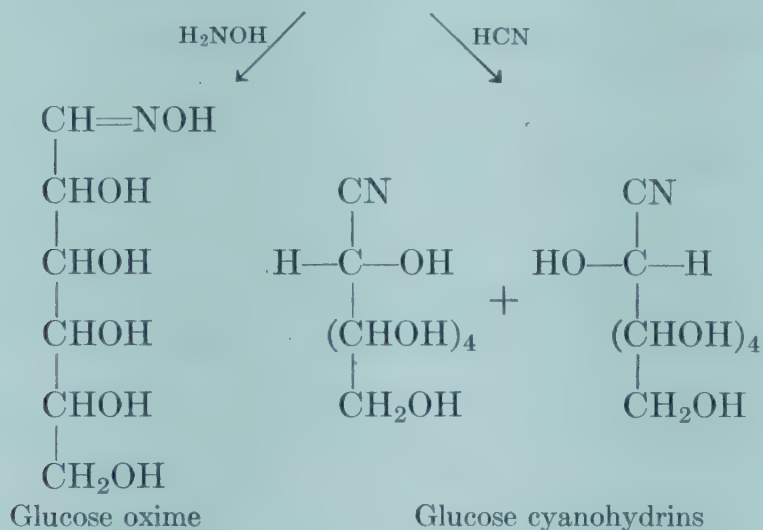
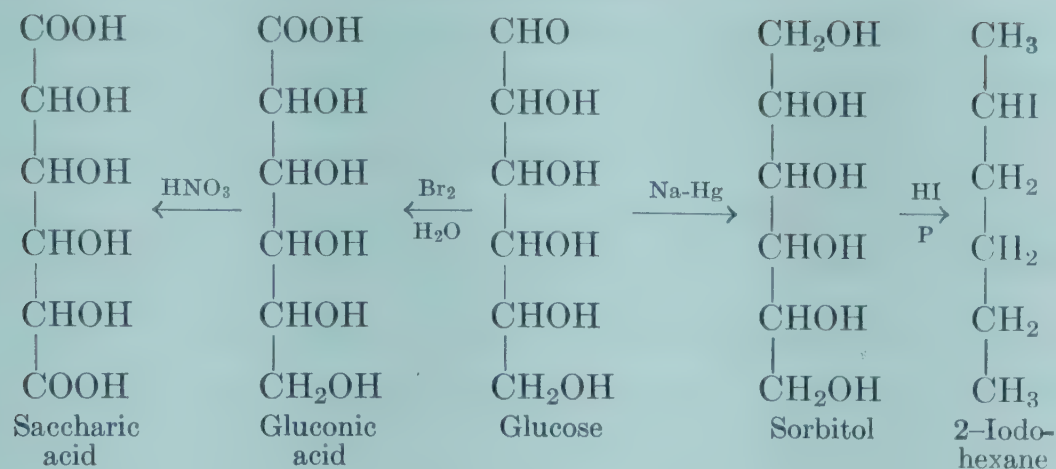
In accordance with these reactions glucose was given the familiar straight chain formula, the carbons of which are now numbered as indicated.



This structure not only accounts for the known reactions of the compound, but also for its optical activity, since each of the four central carbon atoms is asymmetric.

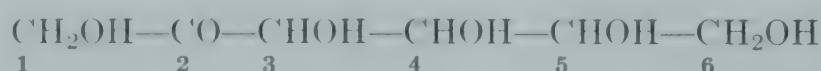
Many other reactions of glucose are those which would be expected of a substance having the suggested structure. It reduces Fehling's solution, reacts with hydroxyl amine to form an oxime and with hydrogen cyanide to form two isomeric cyanohydrins, since the new compound has an additional asymmetric carbon atom. Strong oxidation converts the sugar to the corresponding dibasic acid, saccharic acid.

The addition reaction with hydrogen cyanide, discovered in 1886 by Heinrich Kiliani of Munich, has been of outstanding importance in sugar synthesis. After separation of the two isomeric cyanohydrins they are hydrolyzed separately to the corresponding acids. These compounds readily lose water to give a lactone, and the reduction of the lactone with sodium amalgam yields an aldehyde containing one more carbon than the aldose with which the reaction began. Thus any aldose may be transformed into the next higher aldose, a tetrose yielding two pentoses, these each in turn two hexoses and so on.

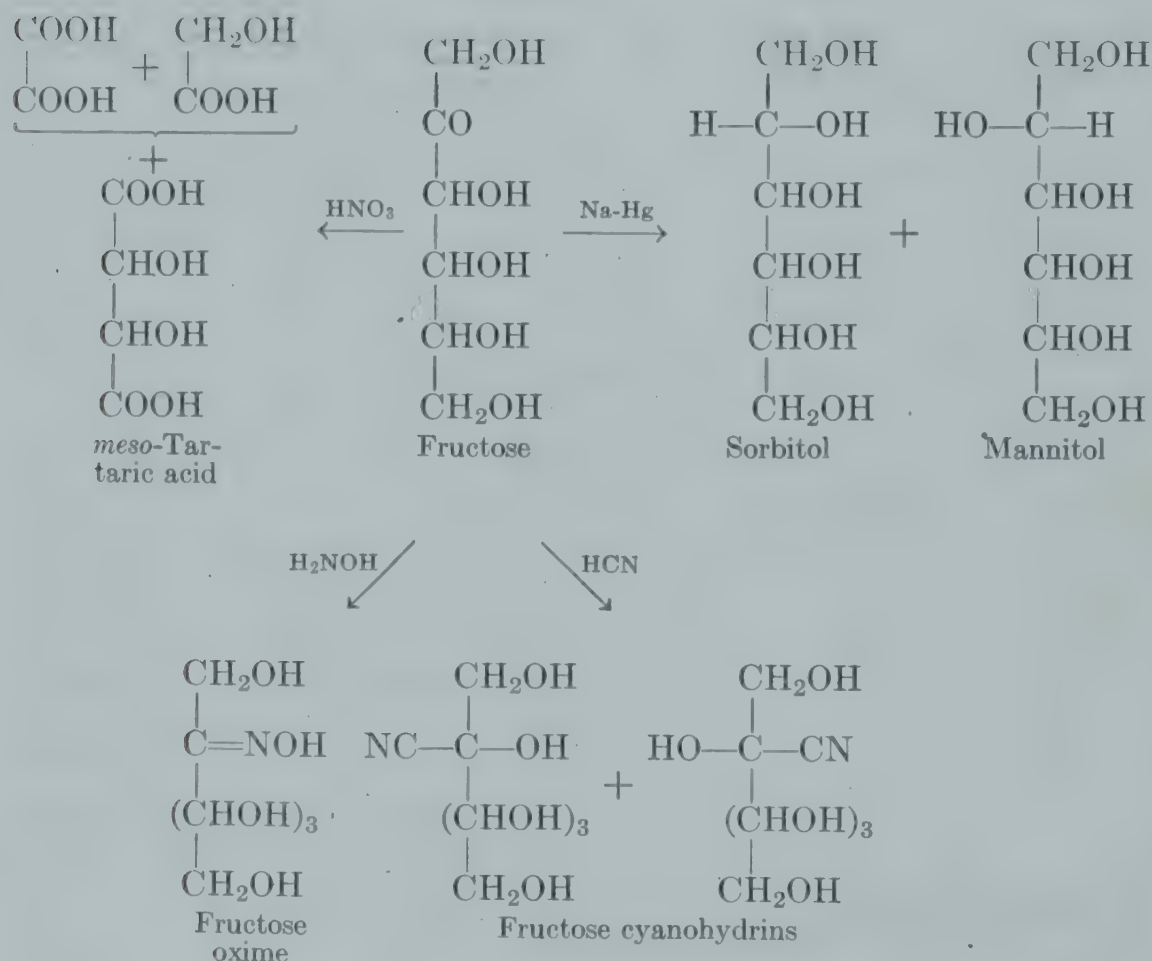


THE KILIANI SYNTHESIS

It may be noted here for comparison that the structure assigned to fructose, which also has the empirical formula $C_6H_{12}O_6$, rests upon evidence similar to that outlined for glucose. Fructose can be reduced to a straight chain hexyl iodide and acetylated to a pentaacetate. But in its response to oxidation it differs from glucose. It is not oxidized at all by so gentle an agent as bromine water, and stronger oxidation with nitric acid breaks the carbon chain. Two different two-carbon acids are formed, glycolic acid ($CH_2OH-COOH$) and its oxidation product oxalic acid ($COOH-COOH$), and also the four-carbon *meso*-tartaric acid. Since fructose gives evidence of the presence of a carbonyl group in its reactions with hydrogen cyanide and with other carbonyl reagents, it is formulated as a penta-hydroxy ketone with the carbonyl group at position 2.



Fructose forms an oxime with hydroxyl amine and a pair of cyanohydrin addition products with hydrogen cyanide. When it is reduced it yields

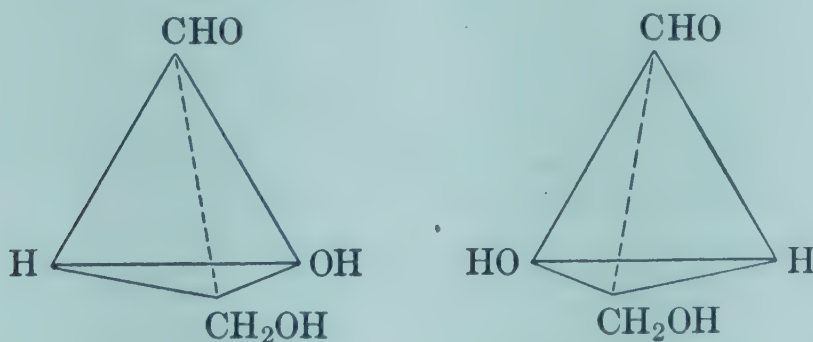


a mixture of two hexahydric alcohols, sorbitol and mannitol, since reduction of the carbonyl group introduces a new asymmetric center at position 2.

STEREOCHEMISTRY OF THE MONOSACCHARIDES

Before going on to study the reactions of glucose, it will be advisable to consider the general optical properties and relationships within the group of monosaccharides.

Projection Formulas. The aldehyde formula for glucose shows it to have four asymmetric carbon atoms, while the corresponding structure for fructose has three. Since the number of possible optical isomers of a compound with n asymmetric carbon atoms is 2^n , there are sixteen possible aldohexoses, of which eight will be mirror images of the other eight. For the ketohexoses there are only eight possible forms, consisting of four isomeric arrangements with their four mirror images or *enantiomorphs*. Emil Fischer worked out for the representation of this confusing group of substances a system of projection formulas derived from the three-dimensional formulas of van't Hoff. Fischer began by actually making tetrahedral models to represent the asymmetric carbon atoms. For the simplest possible optically active hydroxyaldehyde, the two forms of the aldotriose, glycerose or glyceraldehyde, may be represented as follows, the asymmetric carbon atoms at the centers of the tetrahedra being understood.

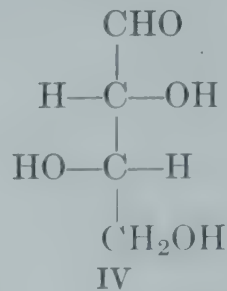
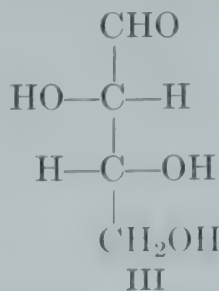
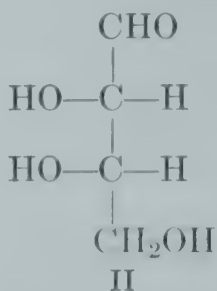
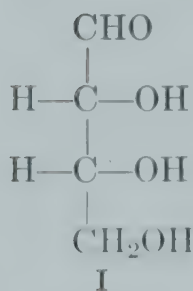


The two forms of glycerose

By convention the aldehyde group of a carbohydrate molecule is written at the top, with the hydrogen and hydroxyl of the asymmetric carbons to right and to left. Fischer therefore proposed such formulas as the following as simple projections on a plane of the models pictured above.

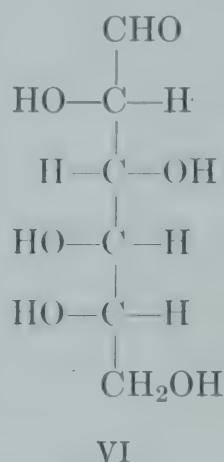
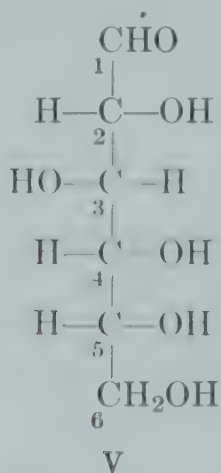


Similar formulas may be written for the tetroses



Formulas I and II are enantiomorphs, as are III and IV. It should be noted that the conventions governing the use of projection formulas preclude their being removed from the plane of the paper. They may be rotated in that plane by rotating the paper itself, but the paper may not be turned over. If this is done, and a formula is read through the paper, it represents, not the original compound but its mirror image. It should also be pointed out that such projection formulas as these take no account of the fact that the carbon atoms are united at an angle of $109^\circ 28'$, and that a "straight chain" of five or six such atoms may in fact be nearly a circle. We shall see later how another type of projection formula takes this into account.

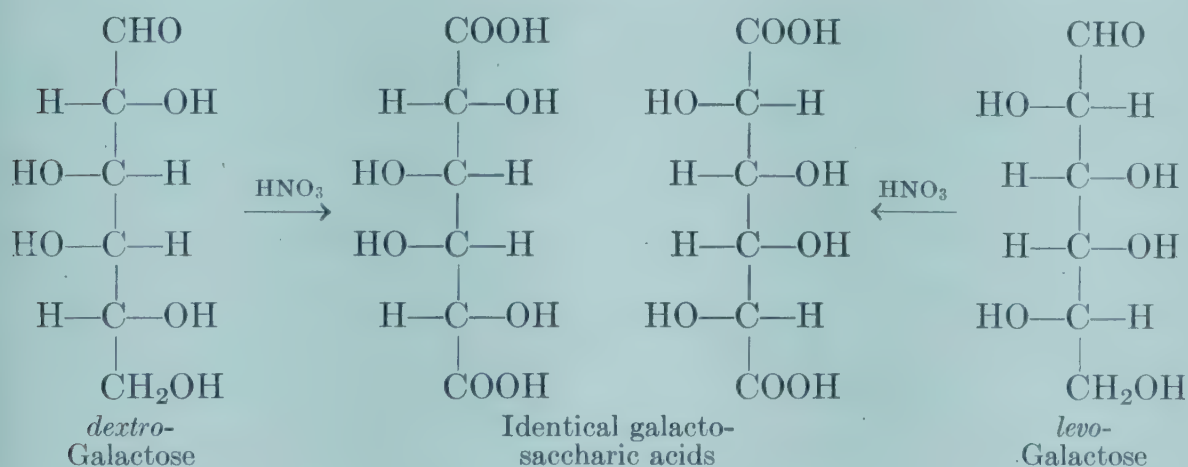
Classification of the Monosaccharides. By a combination of brilliant research and masterly reasoning, Emil Fischer had been able by 1891 to establish the fact that the two enantiomorphs of glucose must have the configurations represented by formulas V and VI. The numbering of the carbon atoms follows the modern convention. In order to assign one of these to the natural, dextrorotatory form of the sugar, it was necessary to set up some frame of reference. There is no known way in which the configuration of a single form can be determined.



Some sort of arbitrary decision must be made, assigning a formula to one of two forms, after which corresponding formulas can be assigned to related substances. Fischer took as his starting point the saccharic acid derived from glucose, named it dextro-saccharic acid and assigned to it one

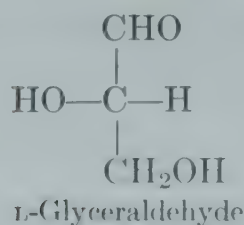
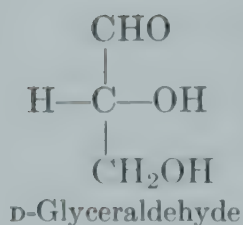
of the two possible enantiomorphic structural formulas. He then worked out the relationships between this substance and a number of different aldohexoses. As a result he divided all the aldohexoses into two series, the one including all those believed to be structurally related to the saccharic acid obtained by oxidation of glucose, the other made up of sugars similarly related to the levo-saccharic acid. On the basis of this classification he assigned to glucose the formula V.

Fischer's idea of dividing all the aldohexoses into two series of mirror images was fundamentally sound, but in choosing the symmetrical saccharic acid as his reference compound he happened to choose badly. For example, when the two enantiomorphic forms of galactose were oxidized to the corresponding dibasic acids, it was found that, because of the symmetry of the galactose molecule, the same acid was obtained from both forms. That the two acids are identical may be seen by rotating the formula of one of them through 90° . Clearly if Fischer's classification



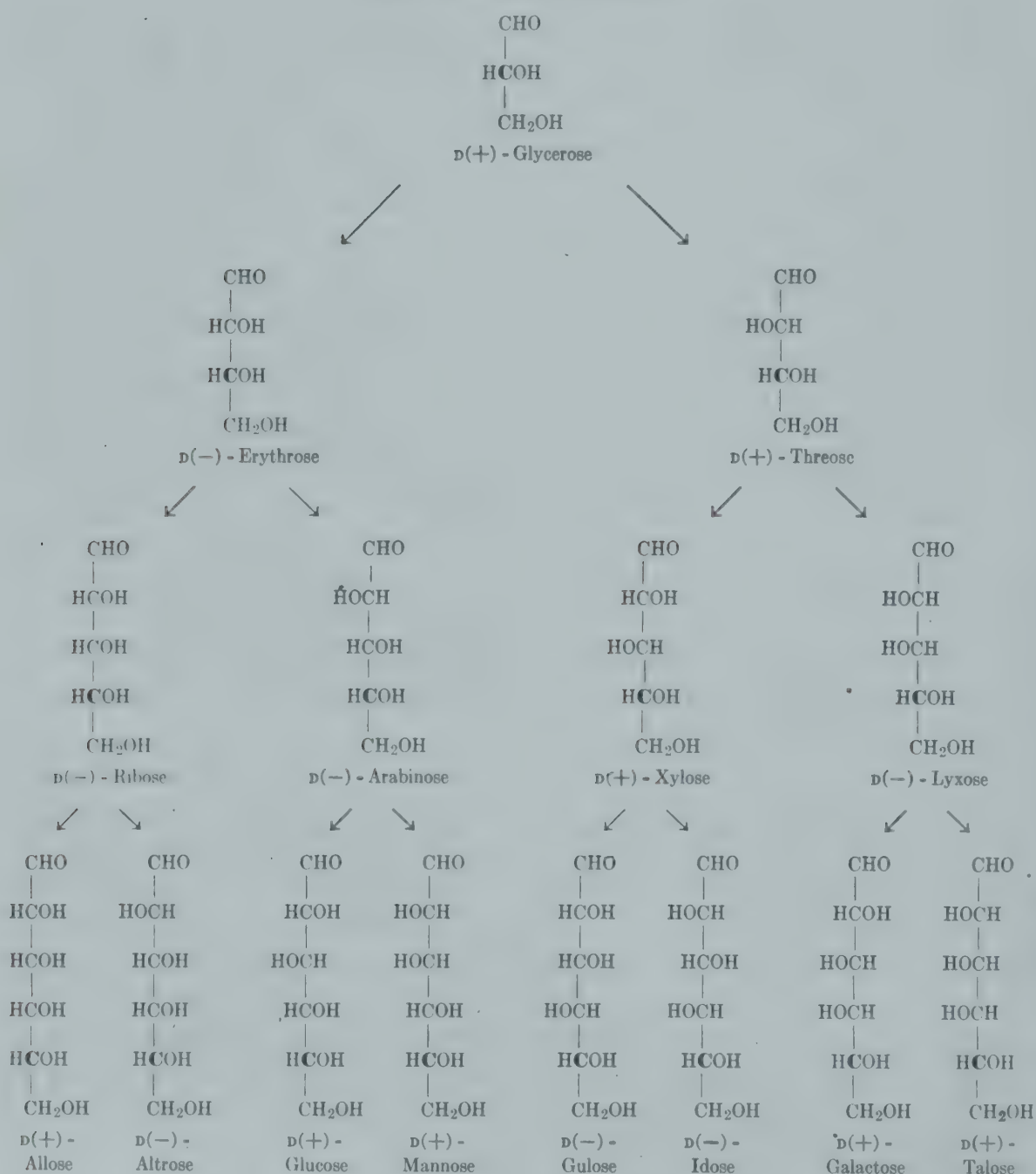
was to have any meaning enantiomorphic forms of a sugar could not both belong to the same series. Similar difficulties with other sugars have led to the use of a different reference compound by means of which the sixteen aldohexoses are now classified satisfactorily into two groups referred to as the **D**-series and the **L**-series.

The system of classification which is now in use was suggested in 1906 by M. A. Rosanoff who was then at New York University. It is possible to think of all the simple aldoses as derived by repeated Kiliani syntheses from the two glyceraldehydes which are the simplest molecules which can be described as "polyhydroxy aldehydes." Rosanoff arbitrarily assigned to the dextrorotatory form of glyceraldehyde the projection formula with the secondary hydroxyl group written on the right, and classified as members of the **D**-series all sugars which could theoretically have been synthesized from this compound. It should be noted that the letters **D** and **L**, which are always to be read "dee" and "ell," are assigned to given sugars regardless of their actual signs of rotation.



The cyanohydrin reaction introduces a new asymmetric carbon atom, hence each step in the progressive building up of the higher sugars gives rise to a pair of stereoisomers. Since however the part of the molecule below the new carbon 2 is identical in both isomers they are not enantiomorphs. Compounds of this sort, having several asymmetric centers but

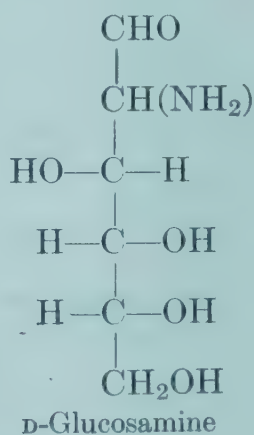
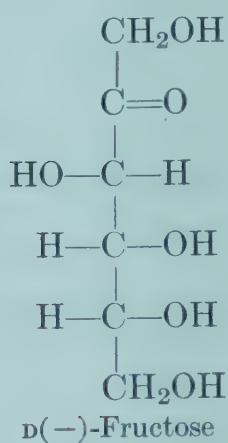
THE D-SERIES OF ALDOSES



differing only in the stereochemical configuration about the carbon adjacent to the reducing or other functional group are known as *epimers*.

The chart on page 92 shows the relationships which Rosanoff traced between D-glycerose and the tetroses, pentoses, and hexoses which could conceivably have been synthesized from it. As it happens nearly half of the sugars in this D-series rotate plane polarized light to the left, and so it is customary to indicate the direction of rotation by a plus or a minus sign. Thus the levorotatory enantiomorph of glucose is designated L(−)-glucose, while the form of arabinose which is levorotatory belongs to the D-series and is therefore D(−)-arabinose. As the sugars of one series are built up from glycerose the only constant factor is the arrangement of hydrogen and hydroxyl around the original asymmetric carbon atom. Thus all members of the D-series have, according to the conventions governing the writing of such projection formulas, the hydroxyl group on the penultimate carbon written to the right. This carbon is the one shown in heavy type in the chart.

Since the Rosanoff classification depends only upon the configuration about this one carbon, it is easily extended to include compounds other than the aldoses. Fructose for example, in spite of a high levorotation, belongs to the D-series, as does the related amino sugar, glucosamine.

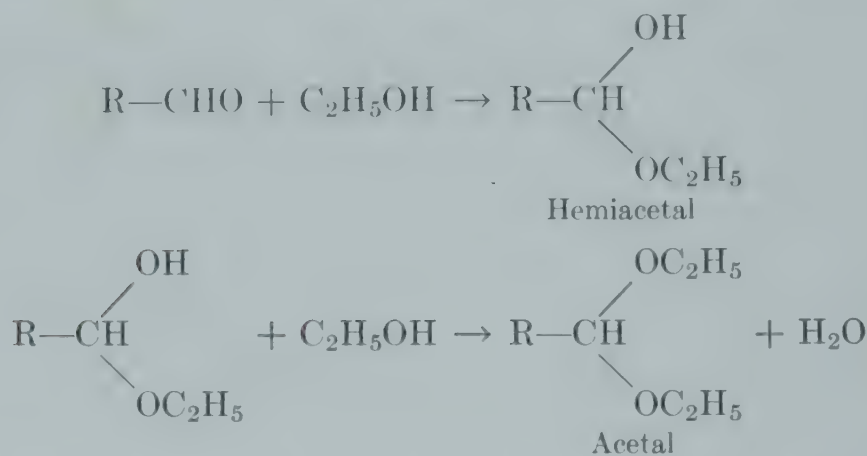


RING STRUCTURE OF THE MONOSACCHARIDES

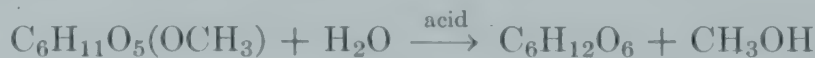
As was briefly indicated above, many of the reactions of the simple sugars can be adequately explained on the assumption that they are straight chain compounds containing a carbonyl group. But from the beginning there were certain facts which could not be reconciled with this formulation. In the first place the reducing power of glucose solution is far in excess of that to be expected of a compound with but one aldehyde group, and at the same time other properties which such a compound should show are absent. It does not restore the color to Schiff's reagent, and the dry compound is strangely inert to atmospheric oxygen. If the old formula were correct the pentaacetate should still react as an aldehyde, which it does not do. Finally there was for years the mysterious

"mutarotation," first noted in 1846 with glucose solutions. Most optically active substances when put into solution exhibit at once the rotation which is characteristic. This is expressed as a *specific rotation*, defined as the rotation caused by a 1 dm. column of a solution containing 1 g. of solute per ml.² But when the specific rotation of a freshly prepared sugar solution is measured, it is found to change gradually during the first few hours, finally reaching a constant value. With ordinary glucose the initial specific rotation, $[\alpha]_D +111^\circ$, drops slowly to $+52.5^\circ$ if the solution is allowed to stand. Other sugar solutions undergo similar changes in rotation for which there was no explanation so long as they were formulated as straight chain compounds.

Evolution of the Modern Formula for Glucose. In 1893 Emil Fischer attempted to prepare an acetal by heating glucose with methyl alcohol acidified with hydrochloric acid. This is a typical aldehyde reaction in which one molecule of the aldehyde reacts with two of alcohol. But the



reaction between glucose and methyl alcohol proved not to follow a typical path. Fischer isolated from the reaction mixture a crystalline product which melted at 165° , had a specific rotation of $+157^\circ$, but did not analyze as an acetal. Its empirical formula was $\text{C}_7\text{H}_{14}\text{O}_6$ which, since it proved to have but one methoxyl group, may be written $\text{C}_6\text{H}_{11}\text{O}_5(\text{OCH}_3)$. Fischer called the compound a methylglucoside since, like the naturally occurring glucosides, it is readily hydrolyzed in acid solution.

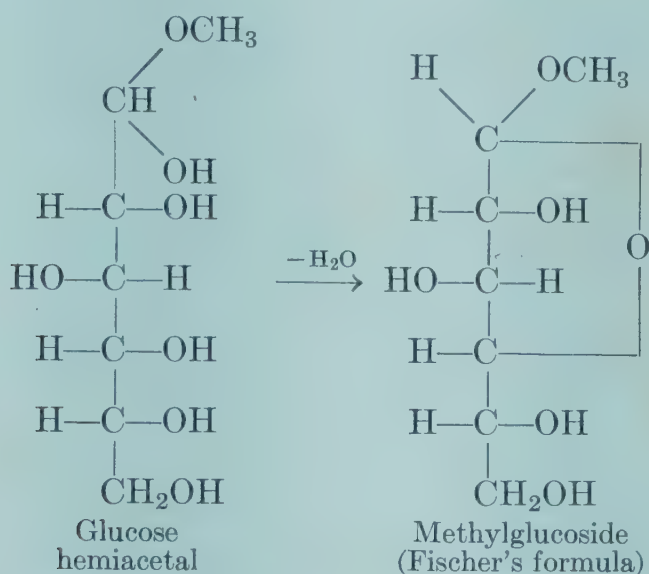


² Since in practice it is seldom possible to make a solution as concentrated as the specified one, the actual rotation is measured with a more dilute solution and the specific rotation calculated from the formula:

$$[\alpha]_D = \frac{\alpha v}{lw}$$

in which α = observed rotation, v = volume of the solution in ml., l = length of the tube in dm. and w = weight of the solute in g. The subscript D indicates that the light used is the yellow sodium light corresponding to the D line in the spectrum. Occasionally the wave length of the light is given in Ångstrom units.

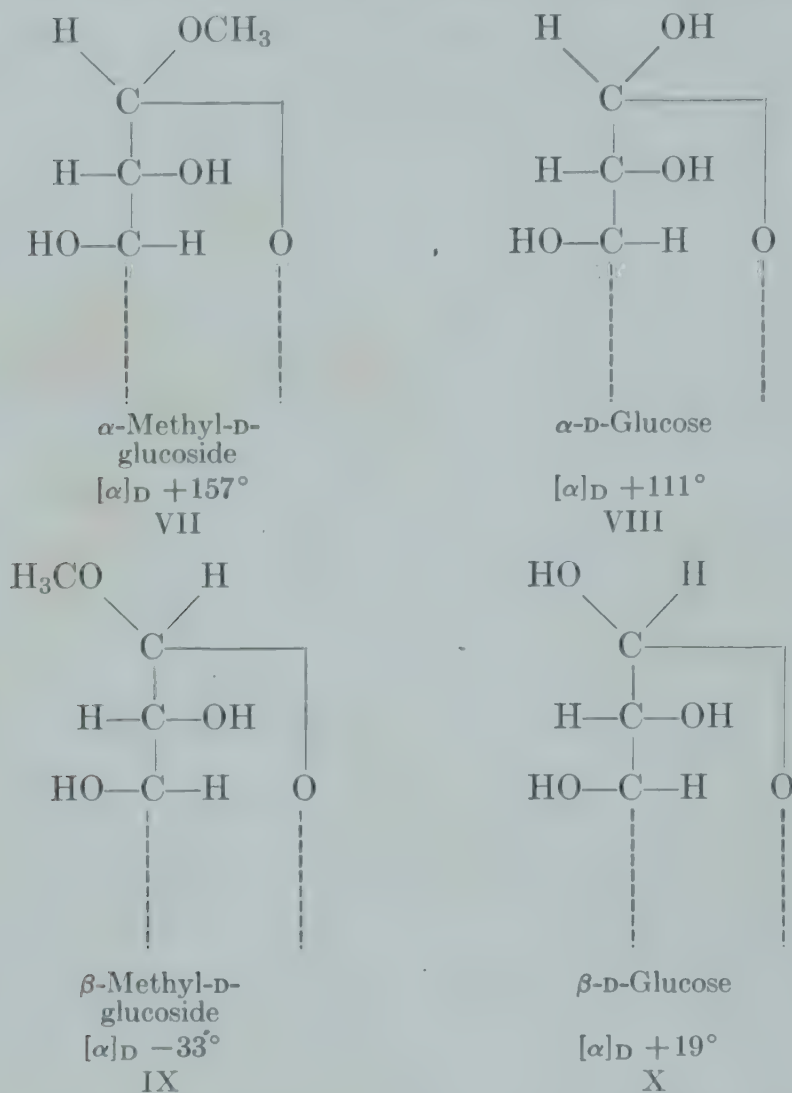
Fischer suggested that in this reaction the aldehyde group reacted with one hydroxyl group of methyl alcohol and with a second hydroxyl group of its own chain. This can be followed if the reaction is written in two steps, in the first of which the sugar reacts to form a hemiacetal. The second step then gives rise to a ring compound through a splitting out of water between the new hydroxyl group on carbon 1 and an hydroxyl group of the sugar molecule. Fischer wrote the product of this second step, erroneously as it proved, as a five-membered ring.



In the paper in which Fischer reported on the methylglucoside he noted that in this compound carbon 1 had become asymmetric, and that there should therefore be two forms derivable from glucose. In the following year Alberda van Eckenstein at Amsterdam isolated the second methylglucoside and called it β -methylglucoside to distinguish it from Fischer's α -methylglucoside. The new compound melted at 104° and had a much lower specific rotation (-33°) than the α -form.

Fischer had been careful to point out that a ring structure for the glucoside did not necessarily indicate a ring structure in the parent sugar. But before the end of 1895 Charles Tanret, a pharmacist in Paris, had shown that glucose itself exists in two modifications. By crystallizing glucose at temperatures above 98° he had obtained an isomeric form, now known as β -glucose, which had an initial specific rotation of $+19^\circ$, contrasted with the initial rotation of $+111^\circ$ given by ordinary or α -glucose. There were thus shown to be one form of glucose with a high initial rotation and a second form with a much lower rotatory power; and these invited comparison with the two methylglucosides with their very different specific rotations. One of Fischer's students, E. Frankland Armstrong (1878–1945), then at the Central Technical College in London, established the relationship between the sugars and the glucosides when he showed that enzymatic hydrolysis of α -methylglucoside resulted in formation of

glucose with a high initial specific rotation, while the β -methylglucoside gave rise to glucose with a low initial specific rotation. Omitting for the moment the question of ring size, the formulas VII to X bring out the relation between the two glucosides and the corresponding sugars.



Such pairs of compounds as α - and β -glucose, in which the asymmetry at carbon 1 depends on ring formation are known as *anomers*, and in consequence the first carbon of the sugars is often referred to as the anomeric carbon.

Projection Formulas of Anomeric Forms. The results just outlined established the fact that D(+)-glucose exists in two anomeric forms. It has since been shown that all the aldo- and ketohexoses have similar ring structures, and that there are therefore 32 possible aldoses and 16 ketoses. The convention which governs the writing of projection formulas for each pair of compounds was proposed by C. S. Hudson.³ For pairs of α - and

³ During the years in which the fine structure of the sugars was being worked out, Claude S. Hudson (1881-1952) of the U. S. Public Health Service was the outstanding American in the field.

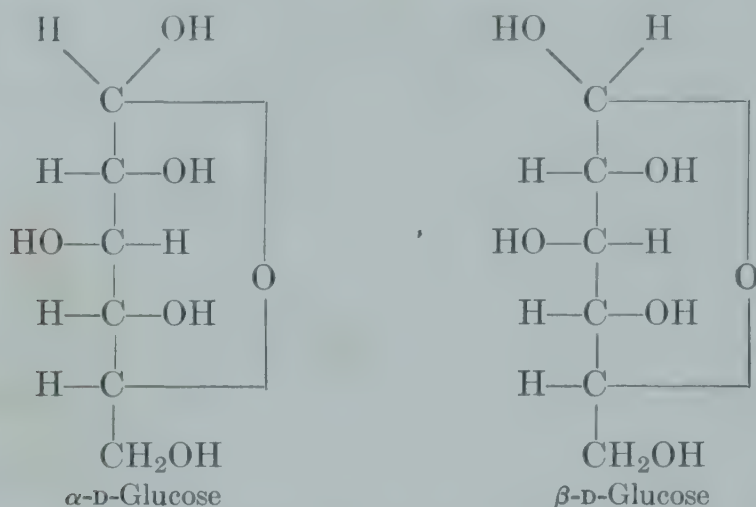
β -isomers in the D-series, the one with the higher rotation is called the α -form, and in its projection formula the hydroxyl or methoxyl group is written on the right. In the L-series there are of course similar pairs of isomers, the enantiomorph of α -D-glucose, $[\alpha]_D +111^\circ$, being α -L-glucose, $[\alpha]_D -111^\circ$. This means that in the L-series the isomer with the lower specific rotation is the α -form, and its hydroxyl group is written on the left. Recent work both chemical and physical has shown that the formulas based on these rules do in fact express correctly the relations in space of the various hydroxyl groups.

Since linkages of the type found in the methylglucosides are extremely common and very important, it seems appropriate to note here one or two matters of general application. Armstrong found that hydrolysis of an α -glucoside could be catalyzed by the enzyme maltase, present in germinating barley, but that this enzyme does not act upon a β -isomer. The latter was however readily hydrolyzed under the influence of another enzyme, emulsin, which can be extracted from bitter almonds. Later work has shown that maltase acts specifically upon any α -glucosidic link, while emulsin catalyzes hydrolysis of β -glucosides. Another generalization which is sometimes useful in distinguishing between α - and β -isomers, concerns their relative specific rotations. The differences found in the optical rotations of the α - and β -isomers of glucose and the methylglucosides, sets the pattern for such differences between other α - and β -forms. In general, the α -configuration leads to higher positive, or lower negative, rotations than result from a β -arrangement. While such a generalization may not be pressed too far, it does frequently offer confirmatory evidence for a given structure.

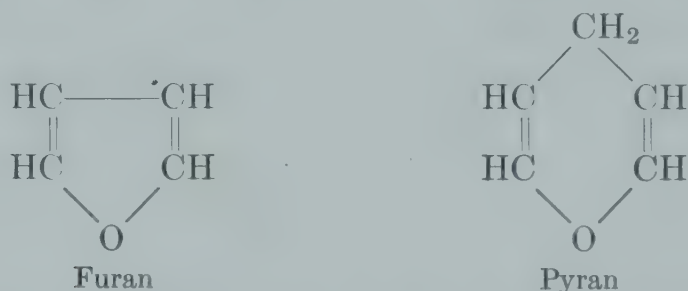
Ring Size. For many years the formula of glucose was written as Fischer had formulated the methylglucoside, with a five-membered ring. As a matter of fact it was impossible to prove what its size might be until after the discovery of some procedure which would block the hydroxyl groups with some stable linkage. It was of course possible to acetylate them, but the ease with which the acetyl groups were removed by hydrolysis made them unsuitable for structural studies. The method of methylation, yielding not ester but ether linkages, opened the way for the work of W. N. Haworth⁴ and C. S. Hudson which finally established the structure as that of a six-membered ring, with oxygen linking carbons 1 and 5. This beautiful chapter of structural organic chemistry would take

⁴ When Sir Norman Haworth (1883–1950) began his investigations in the field of carbohydrate chemistry about 1912 at St. Andrews University, Thomas Purdie was still active, though retired, and J. C. Irvine was Director of the Chemistry Department. This was a conjunction of stars of the first magnitude, which gives to St. Andrews a very special place in the history of structural sugar chemistry, even though the last twenty-five years of Haworth's professional life were spent at the University of Birmingham. In 1937 Haworth shared with Paul Karrer of Zürich the Nobel Prize in Chemistry.

us too far afield, though we shall shortly see how the methylation procedure was used in elucidation of the structures of other sugars. For the moment we will simply revise the Fischer formula to indicate that crystalline glucose has been shown to exist as a six-membered ring. On pages 109-110 a modern proof of this ring formulation is outlined.

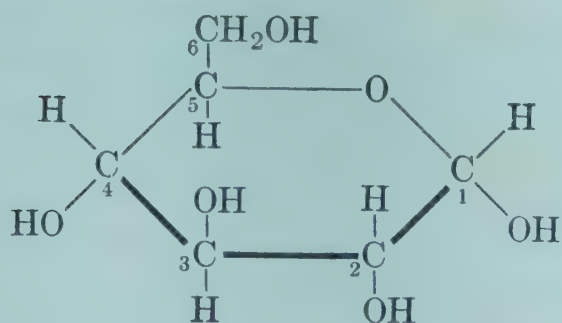
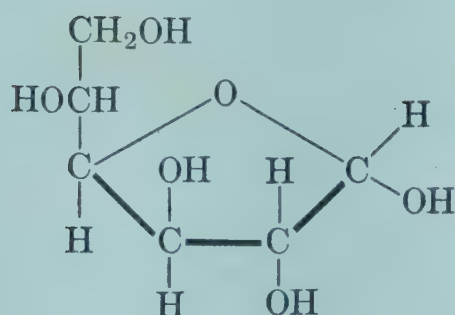
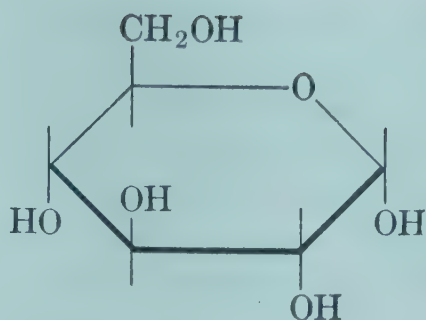
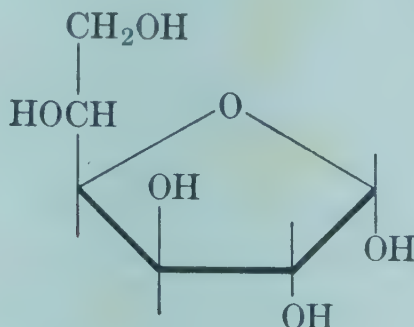


Ring Projections. Although the stable form of the free hexoses and pentoses contains a six-membered ring, there are compounds in which the oxygen bridge goes to a γ -carbon. For example, Fischer isolated in 1914 a third, very labile methylglucoside which was truly called a " γ " form, since its oxygen bridge proved to go to the γ -carbon. Likewise the sugar acids such as gluconic acid form stable γ -lactones, and in certain compounds fructose occurs as a five-membered ring. In order to distinguish these various forms names are given which relate a sugar to one or the other of the simple heterocyclic compounds, furan and pyran.

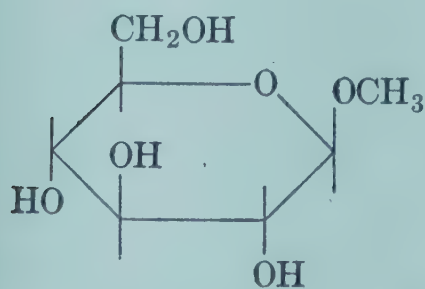
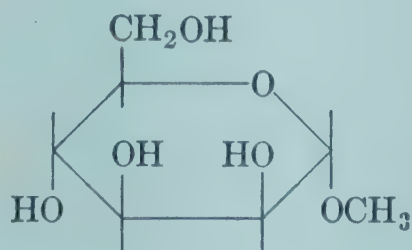
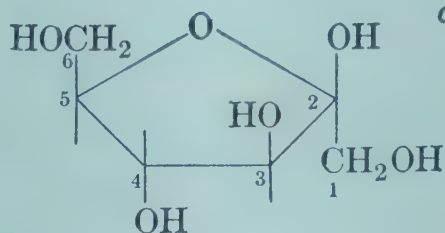


Sugars with six-membered rings are known as *pyranoses* and the corresponding glucosides as *pyranosides*. Similarly the sugars and glucosides with 1-4 links are named respectively *furanoses* and *furanosides*.

It is often convenient to indicate the relation between the various atoms in the sugar more graphically than is possible with the type of straight chain formulas which have been used up to this point. Haworth achieved this by use of a projection formula in perspective, which emphasizes the ring structure. These are now commonly used with both the carbons and the hydrogens omitted as shown on the right, since for clearness it is necessary to know only the disposition of the hydroxyl groups.

 α -D-Glucopyranose α -D-Glucofuranose

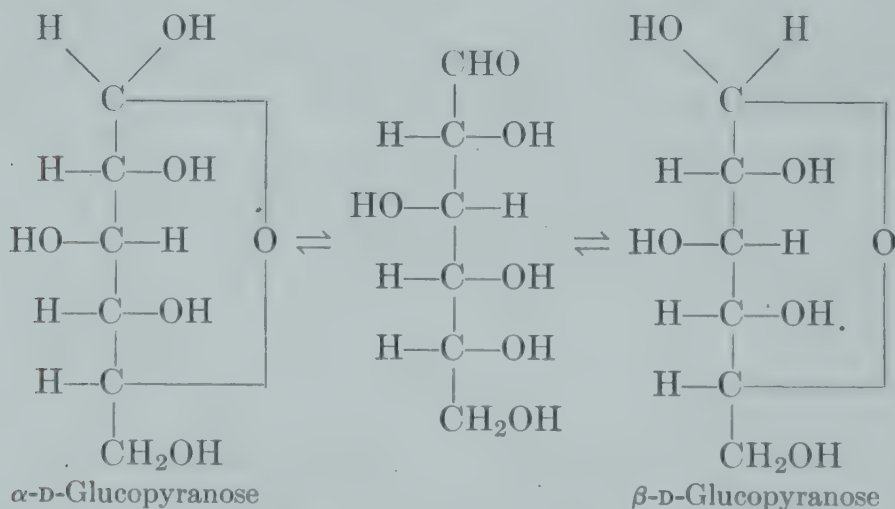
The heavy lines emphasize the fact that we are looking at what is in effect a drawing of a space model in which four or five carbons and an oxygen form a ring in a single plane at right angles to the plane of the paper. The attached groups are disposed above or below this plane. Those groups which were written on the right in the straight chain formula appear below the plane of the ring, while those which were written on the left appear above it. Further illustrations given below use only the simplified formulations and omit the heavy ruling which was first used to make the perspective more readily visible.

 β -D-Methylglucopyranoside α -D-Methylmannopyranoside β -D-Fructofuranose

The position of the primary alcohol group in the pyranose forms is determined by the position of the hydroxyl on carbon 5. If this hydroxyl group lies to the right in the Fischer formula the primary alcohol group extends above the plane of the ring, but it is below the plane when the adjacent hydroxyl is on the left. This would appear to place the hydrogen attached to carbon 5 on the wrong side of the chain. But with an actual model of the aldehyde form it is found that in order to bring the hydroxyl group on carbon 5 into such a position that it can be linked by an oxygen bridge to carbon 1, the chain must be twisted so that the hydrogen crosses to the opposite side of the ring plane.

Value of the Ring Formula. It was pointed out on page 93 that there were certain objections to the aldehyde formula for glucose. This does not however change the fact that glucose in solution does react with nearly all aldehyde reagents. Before the ring structure can be accepted it must be reconciled with this fact and be shown to explain also the various anomalous properties referred to above.

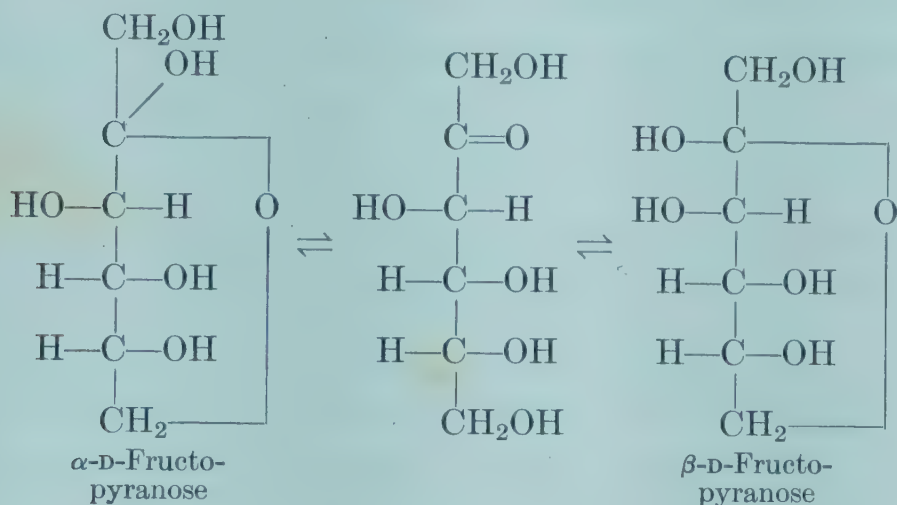
There is a certain amount of indirect evidence that when crystalline glucose goes into solution a small amount of the ring compound shifts a proton and takes on the aldehyde structure. The shift is reversible and the proton returning to its position on the anomeric carbon may take either the α - or the β -configuration. Eventually a mixture results in which the α -glucose is in equilibrium with the β -form through the formation of the intermediate open chain aldehyde. The actual amount of the reducing



compound in a solution of glucose is very small, and indeed for most sugars with the exception of ribose makes up less than 1 per cent of the total. The fact that there is at any one time so low a concentration of the reducing form accounts for the unexpected slowness of some of the aldehyde reactions of glucose. Thus when an aldehyde reagent is added to the equilibrium mixture of the three forms, it upsets the equilibrium by reacting with whatever aldehyde is present. In accordance with the Law of Mass Action more aldehyde is formed, and this in turn reacts with the

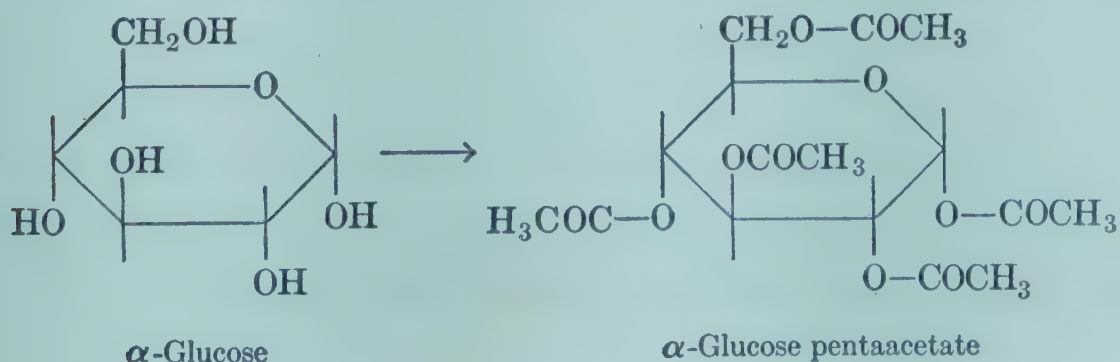
reagent. Eventually all of the ring forms are transformed into the straight chain compound, which is continuously removed. Thus the glucose finally reacts as if all of the sugar had been present in the aldehyde form, giving rise to cyanohydrins or oxime. For such reactions the simplest formulation uses the aldehyde formula only, its relation to the ring forms being understood.

Fructose also occurs as a ring compound when in the crystalline condition and gives rise to an equilibrium mixture in solution.



This behavior of the labile sugar molecules in solution explains also why they exhibit mutarotation. Ordinary glucose is the α -form, having a specific rotation $+111^\circ$. As it slowly comes to equilibrium with the β -form, $[\alpha]_D +19^\circ$, the specific rotation of the mixture falls until equilibrium is established and with it the equilibrium specific rotation of the sugar, $+52.5^\circ$. Similarly the β -form of glucose shows an upward mutarotation to the equilibrium value.

Finally, the absence of a reducing group in the glucose pentaacetate is to be expected if almost all of the sugar is present in solution in a non-reducing form. The acetylating agent attacks the five hydroxyl groups in the molecule, and once the anomeric hydroxyl has been acetylated the ring form is stabilized. An atom as small as the hydrogen atom can easily slip back and forth between carbon 1 and carbon 5, but this is not true of the large acetyl group. When the α -form of crystalline glucose reacts



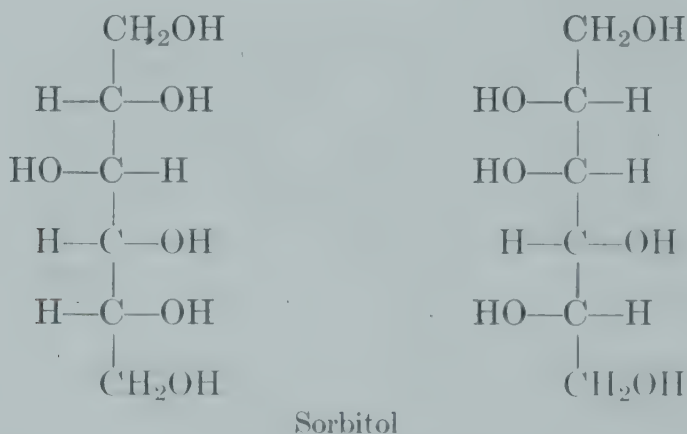
with acetic anhydride in the presence of a catalyst such as zinc chloride, the reaction which takes place yields chiefly a glucose pentaacetate in which one of the five acetyl groups is attached to carbon 1. However, from some such reaction mixtures there has been isolated, in addition to the cyclic, nonreducing pentaacetates, very small amounts of a reducing form. This is a direct demonstration of the presence in the sugar solution of a small amount of the straight chain aldehyde form.

CHEMISTRY OF THE MONOSES

As indicated above, many of the reactions of the monosaccharides can be satisfactorily explained by the use of simple straight chain formulas, the aldoses reacting by virtue of their aldehyde group and the ketoses reacting as ketones.

1. Reduction. It has already been noted that treatment of glucose with sodium amalgam yields the expected hexahydric alcohol, sorbitol. Similar reduction of fructose gives rise, as would be expected from its straight chain formula, to two alcohols which differ only in the configuration about the second carbon. The fact that one of the two is the same sorbitol which is formed in the reduction of glucose demonstrates that the configuration about the other asymmetric centers at carbons 3, 4, and 5 are identical in glucose and fructose.

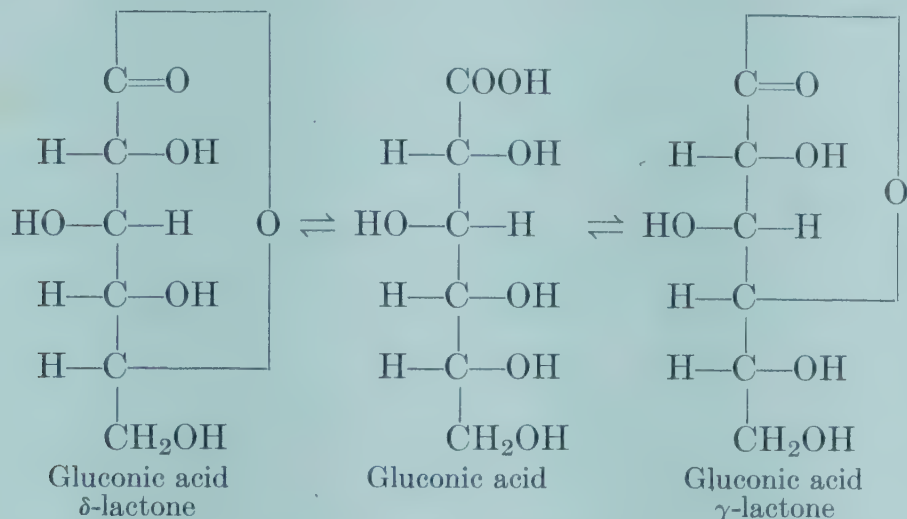
It should be noted that with such symmetrical compounds as the hexahydric alcohols and the dibasic sugar acids, the D- and L-nomenclature has no meaning. For example, sorbitol is a D-compound if written with one primary alcohol group at the top, but is a member of the L-series if the formula is inverted.



The hexahydric alcohols, or hexitols, are systematically named by substituting -itol for -ose in the names of the corresponding sugars. For some compounds the older names persist, as with sorbitol which was discovered in the berries of the mountain ash (*Sorbus aucuparia* L.).

2. Oxidation. When any one of the aldoses is oxidized gently with bromine water a monobasic acid is formed. These are all named, as is the acid related to glucose, by substituting for -ose in the name of the sugar, the suffix -onic. Thus D-galactose forms D-galactonic acid and L-ribose is oxidized to L-ribonic acid.

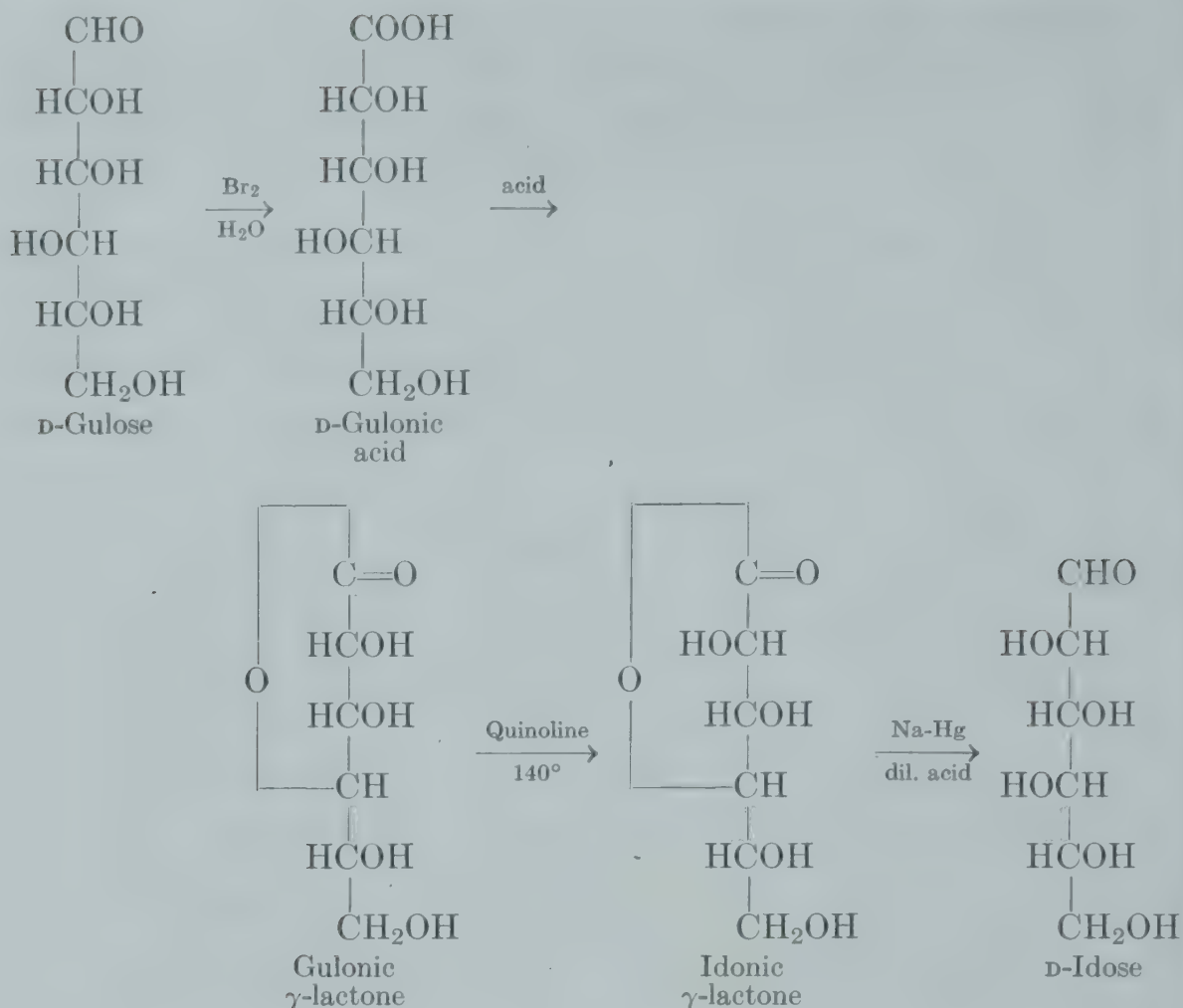
The aldonic acids related to the pentoses and hexoses are α -, β -, γ -, and δ -hydroxy acids, and readily form inner esters or *lactones*. Indeed this elimination of water takes place so readily that the acids scarcely exist as such in solution, but are in equilibrium with their γ - and δ -lactones. Of these the γ -forms are the more stable. The lactones are especially im-



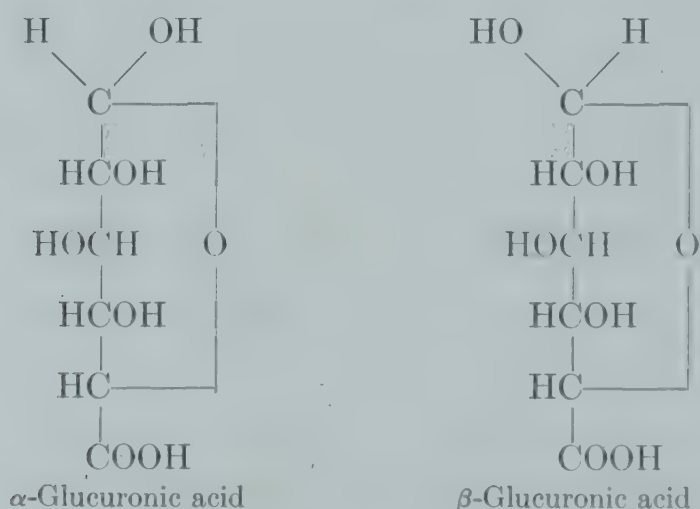
portant because, while the acids are resistant to reduction, the lactones are readily reduced to the corresponding aldoses.

Another reaction of the aldonic acid lactones which has been valuable in the synthesis of sugars is the change of configuration about carbon 2, known as *epimerization*. This takes place when a lactone is heated with aqueous pyridine or quinoline. Part of the original compound is thereby converted to its epimer which can then be separated and reduced to the corresponding aldose by treatment with sodium amalgam. This reaction was used by Emil Fischer in the synthesis from gulose of the epimeric idose (see p. 104).

While the aldonic acids are the only monobasic acids which can be prepared by direct oxidation of the aldohexoses, there is a second series of acids which are of great physiological importance and which are clearly related to the sugars. These are the compounds in which the primary alcohol group of the aldose has become a carboxyl group though the aldehyde group remains unchanged. The acids of this type are known as *uronic acids*, and the individual members are named by use of the suffix -uronic with the root of the name of the corresponding monose. Glucuronic acid is thus the acid of this structure related to glucose, though it is

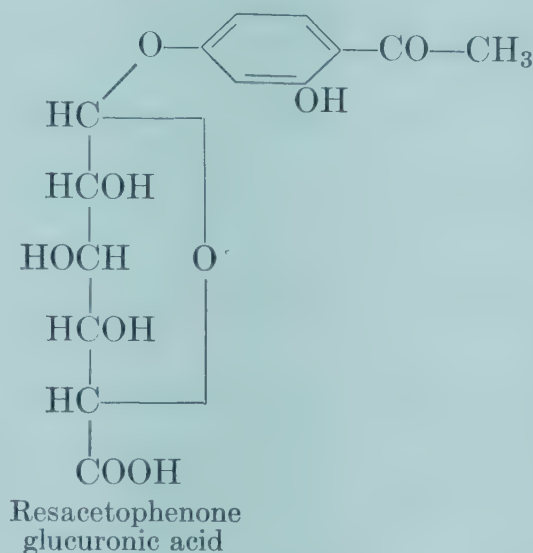
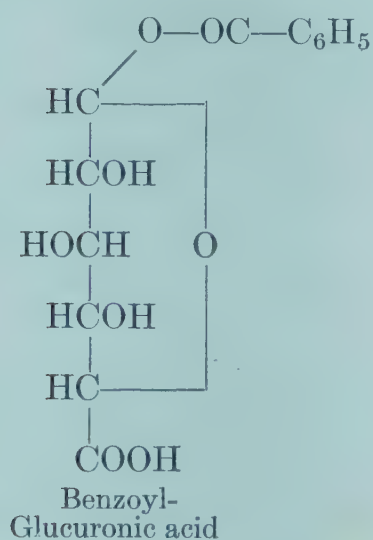


clearly not possible to prepare it by direct oxidation of the sugar. This group of acids is sometimes referred to as the glyceronic acids, the gly- prefix being used here, as elsewhere in sugar chemistry, as a generic term. Thus there are 32 possible *glyceronic* acids of which 2 are *glucuronic* acids.



Compounds of this type are very widely distributed in nature, chiefly in the form of heteropolysaccharides. It has been estimated that 10 to 15

per cent of the organic carbon in the surface soil is in the form of uronic acids, and many plant and bacterial products consist wholly or in part of the uronic acids related to glucose, galactose, or mannose. Glucuronic acid itself serves a special function in animal metabolism because of its ability to form condensation products with various aromatic compounds. Thus administration of various toxic compounds is followed by their excretion in a conjugation product with glucuronic acid. This is true, for example, of benzoic acid and of other aromatic compounds. Although nor-

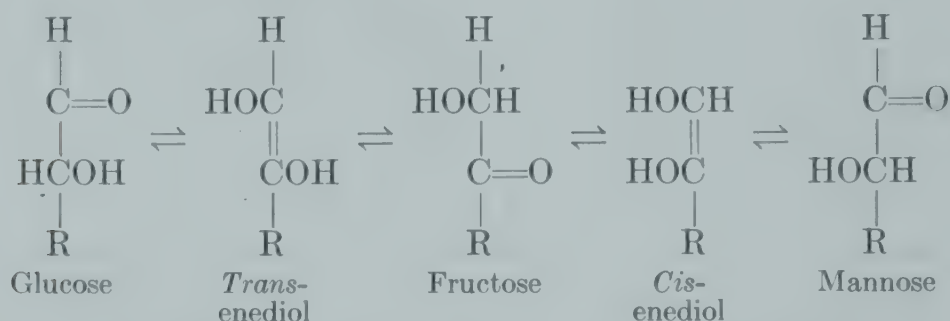


mally only small amounts of the uronic acids are present in the urine, the body is able to adapt itself to the synthesis of much larger quantities when they are needed for detoxication. Whether or not glucose is the immediate precursor is not known.

When glucose is strongly oxidized, as with nitric acid, both terminal groups react and a dibasic acid, saccharic acid, is formed. This name, originally used to designate the acid derived from glucose, has come to be used as a generic term. Thus the isomeric acid derived from mannose is called *manno-saccharic acid*, and most of the other acids of this group are similarly named. The acid related to galactose, on the other hand, has a name of its own, *mucic acid*. Of all the series of dibasic acids only mucic acid is insoluble enough to crystallize from a concentrated water solution, and this fact is made the basis of a test to distinguish galactose from other aldohexoses.

3. Reactions with Alkali. All the monoses, ketoses as well as aldoses, react with those oxidizing agents, like Fehling's solution and ammoniacal silver nitrate, which are ordinarily used to distinguish between aldehydes and ketones. The reason for this anomalous behavior on the part of the keto-sugars is to be found in the great sensitivity of all the simple sugars to alkali. An aqueous solution of glucose, made faintly alkaline with lime water or barium hydroxide, on standing at room temperature will slowly come to an equilibrium in which glucose, fructose, and mannose are

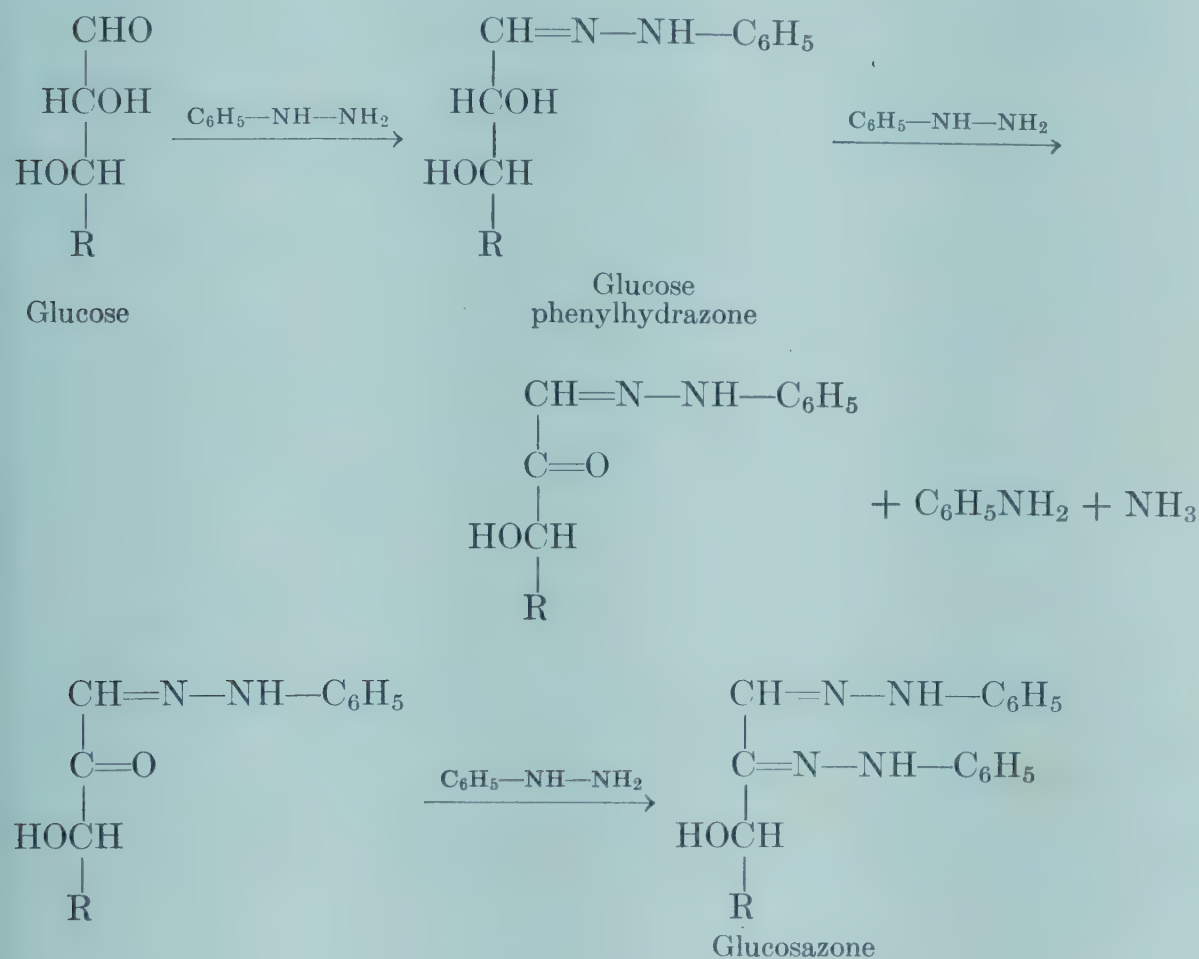
all present. It has been assumed for many years that this transformation takes place through the intermediate formation of an enediol form common to the three sugars. Very recent work with deuterium indicates that the obligatory intermediate is fructose, and that two forms of the enediol compound are involved, one a *cis* form and the other a *trans*. The reversible reactions by which the equilibrium mixture is now believed to form are indicated in the chart, R standing for that part of the molecule which is identical in all three sugars.



In the presence of strong alkali such as is present in Fehling's solution, much more deep-seated changes take place and the carbohydrate molecules are broken into fragments. Some of these have been identified, but the whole process is very complicated and is still quite obscure. There is probably an equilibrium established between degradation products and the results of condensation of some of the fragments with each other. When alkaline copper or silver salts are used to test for the presence of "reducing sugars" the reaction is far from being a simple oxidation of a carbonyl group. Indeed this is one chemical reaction for which no equation can be written, as there are probably dozens of different reaction products. This is indicated by the fact that a given weight of glucose or fructose brings about reduction far in excess of that which could be accounted for by their single carbonyl groups. Under such circumstances, the actual reduction is directly due to all or many of the fragments which originate in the highly labile sugar molecule. This lability in turn is due to the presence of the carbonyl group, since no such reduction takes place with polyhydroxy alcohols or acids. In this sense then, the carbonyl groups are the reducing groups, and a positive reduction test indicates the presence of such groups, free or potentially free, in the sugar molecule.

4. Reactions with Carbonyl Reagents. Even though the reactions with alkaline reducing agents are so complex as to offer little evidence for the aldehyde or ketone structure of the monoses, other carbonyl reagents yield more conventional reaction products. The formation of oximes with hydroxyl amine has already been mentioned, and also the formation of an addition product with hydrogen cyanide.

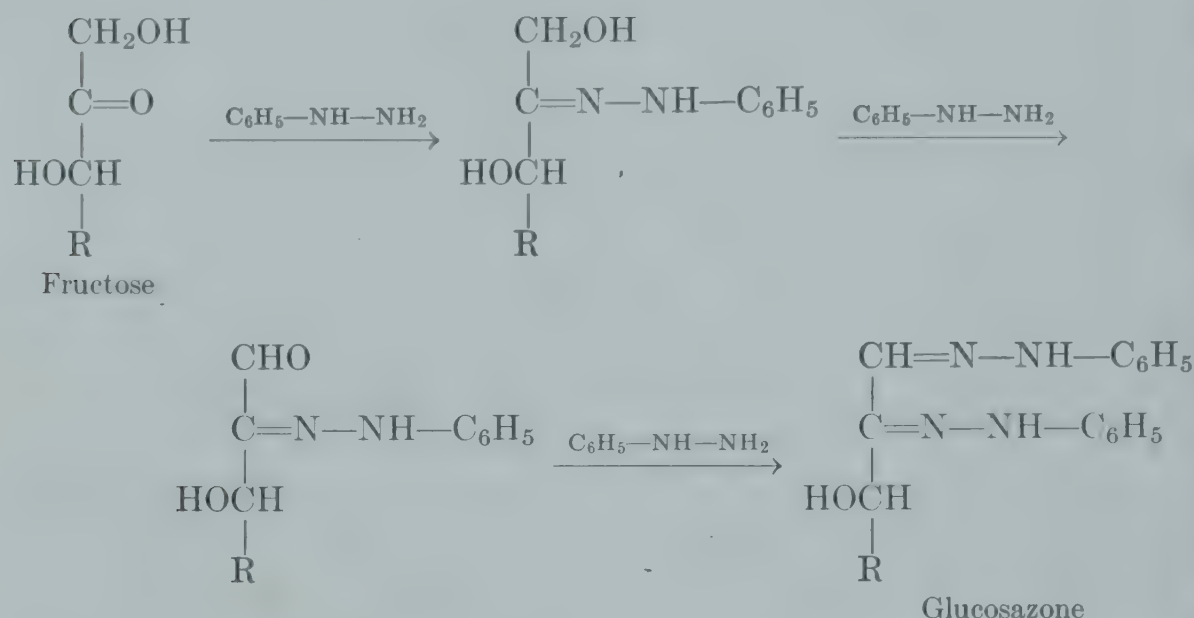
One of the first important contributions to carbohydrate chemistry was the introduction by Emil Fischer of phenylhydrazine as a reagent. The reducing sugars, most of which can be made to crystallize only with great difficulty, react with phenylhydrazine in the presence of acetate buffers to form yellow, beautifully crystalline phenylosazones. The reaction with glucose was formulated by Fischer as follows:



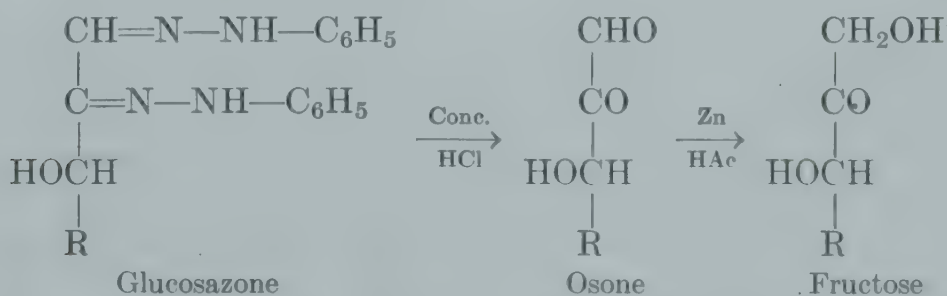
According to this scheme the preliminary formation of a typical phenylhydrazone is followed by an oxidation which yields a 2-keto derivative. This new carbonyl group then condenses with a second molecule of phenylhydrazine to form the crystalline phenylosazone. There is still some uncertainty about the mechanism of the oxidation reaction. The facts are that in the preparation of glucosazone three moles of base are used for every mole of osazone formed, and that of these three, two appear in the products while a third is reduced to yield one mole each of aniline and ammonia. As indicated in the formulation given, Fischer assumed that the phenylhydrazine was itself the direct oxidizing agent. But this mechanism is hard to reconcile with the facts that the free base is not normally an oxidizing, but a reducing agent, while the secondary alcohol group seems far too mild a reducing agent to reverse this tendency. Various alternative

mechanisms have been proposed which involve indirect reduction of the base, but none has yet been generally acceptable.

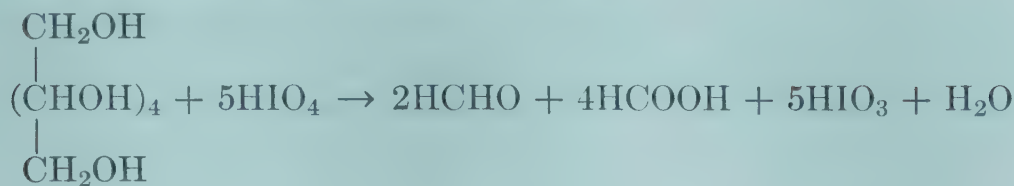
When fructose reacts with phenylhydrazine it yields an osazone identical with glucosazone by a similar series of reactions. This furnishes additional proof that glucose and fructose have identical configurations about carbons 3, 4, and 5.



The phenylhydrazine residues can be removed from an osazone by treatment with concentrated hydrochloric acid, yielding a very reactive compound known as an *osone*. If the osone is then reduced very gently, the aldehyde group reacts preferentially and a ketose results. Thus from the aldose, glucose, by way of its phenylosazone, the ketose, fructose, may be formed.

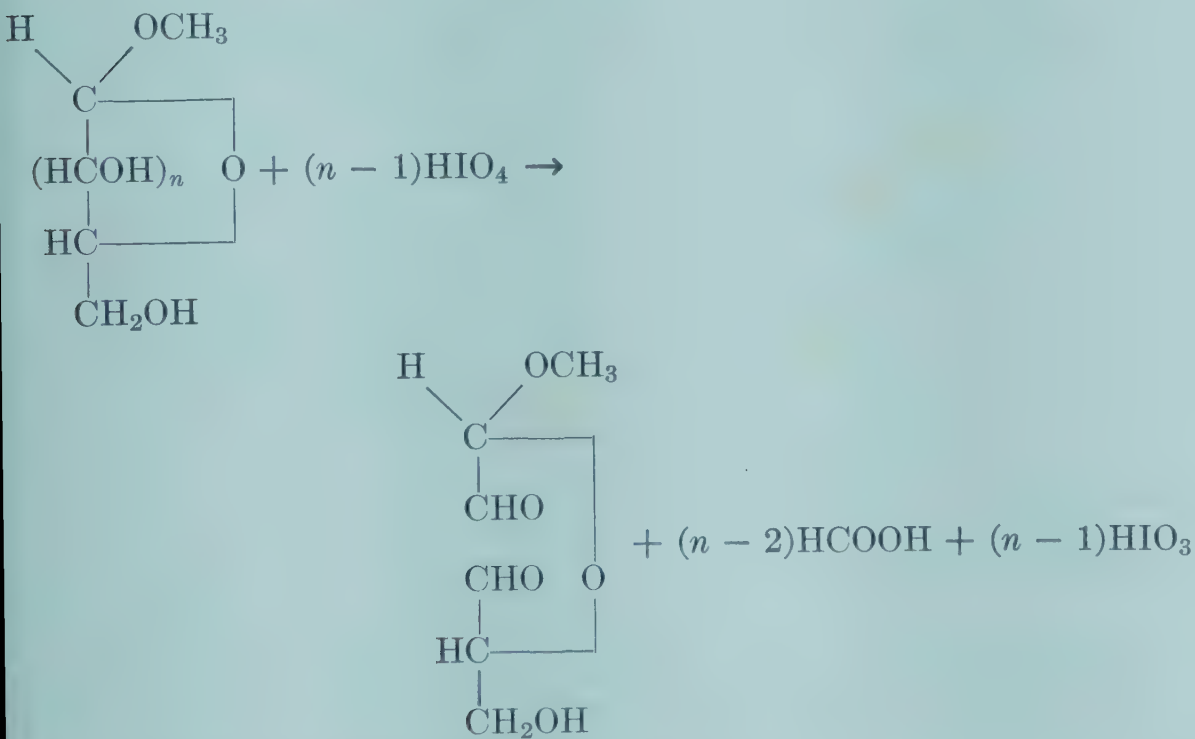


5. Reactions of the Hydroxyl Groups. 1. *Oxidation with Periodic Acid.* In 1928 Malaprade of the University of Nancy reported that periodic acid (HIO_4) oxidizes α -glycols forming formic acid from a secondary alcohol group and formaldehyde from a primary. One mole of the oxidant is used for each glycollic pair oxidized and the reduction product is iodic acid, HIO_3 . When the reaction is applied to a hexahydric alcohol with its five pairs of adjacent hydroxyl groups the following reaction takes place:



The course of the reaction is easily followed since with thymolphthalein as indicator the periodic acid titrates as a dibasic acid and the iodic as monobasic. The formaldehyde is estimated as a crystalline condensation product with dimedone (5,5-dimethylcyclohexane-1,3-dione).

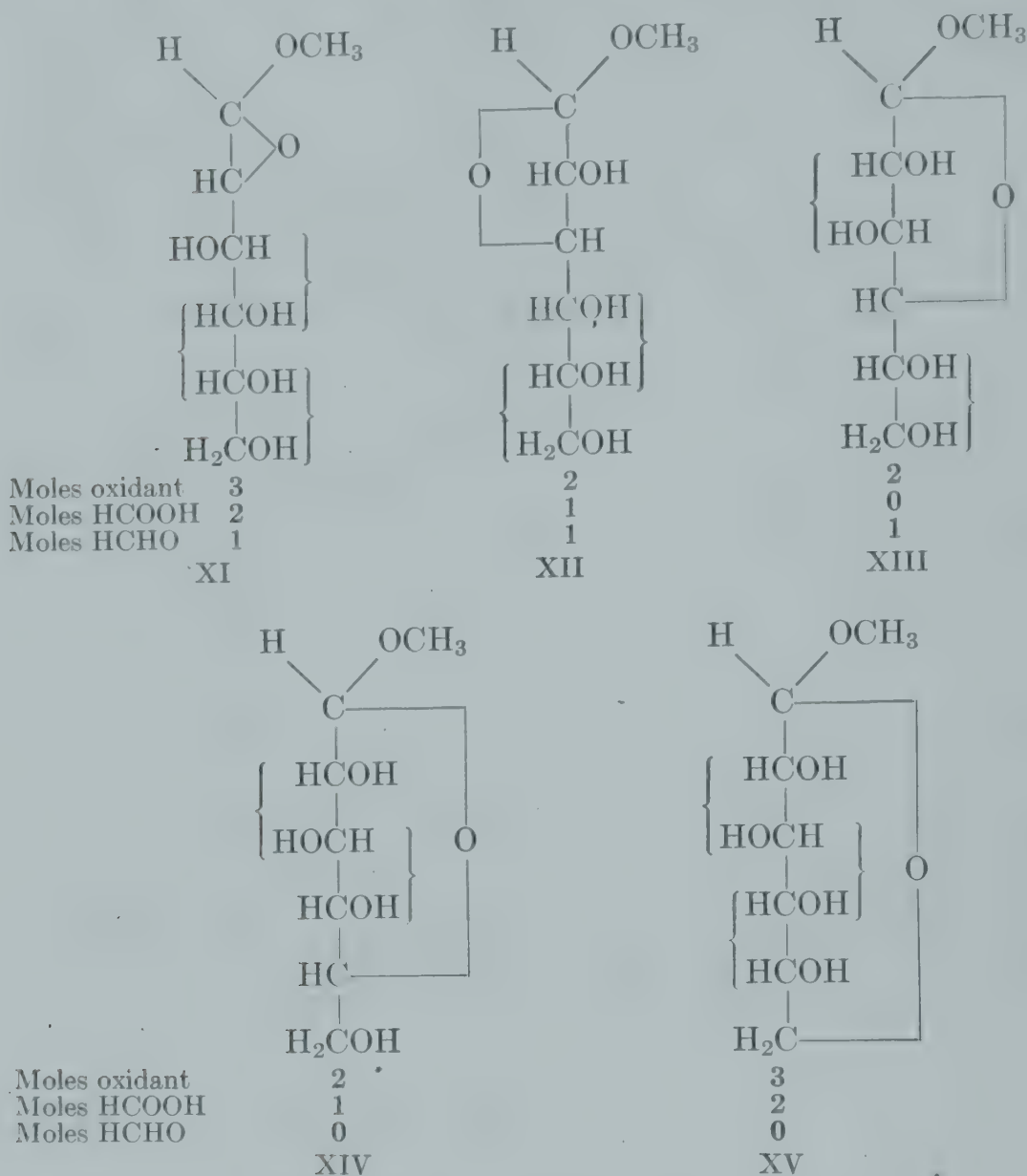
When periodic acid is used for the oxidation of a ring compound such as glucose, it leaves the oxygen bridge intact and breaks the carbon chain between adjacent hydroxyl-bearing carbons. A primary carbinol of a pair yields formaldehyde; a secondary alcohol group between two other carbinols is oxidized to formic acid, but if it has carbinol on only one side it remains attached to the chain and becomes an aldehyde group. The general course of such a reaction is as follows:



When this oxidation procedure was applied to α -methylglucoside it yielded unequivocal proof of the presence of the pyranose ring.⁵ Formulas XI to XV give the ring structures theoretically possible for a methylglucoside. Under each is indicated (a) the moles of periodic acid which one mole of a glucoside of the indicated structure would use, (b) the moles of formic acid, and (c) the moles of formaldehyde which would be formed. These

⁵ The general reaction above as well as the proof of ring structure which follows are quoted with the permission of the publisher from W. W. Pigman and R. M. Goepp, *Chemistry of the Carbohydrates*, Academic, New York, 1948.

amounts all depend upon the number of *pairs* of adjacent hydroxyl groups, and the pairs are bracketed in the formulas.

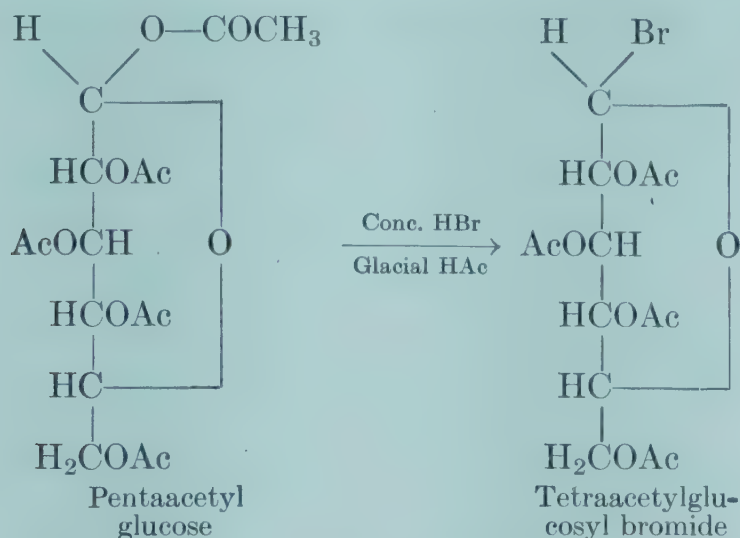


When methylglucoside was oxidized with periodic acid it proved to consume 2 moles of oxidant and to form no formaldehyde, thus establishing the presence of the pyranoside ring as shown in structure XIV.

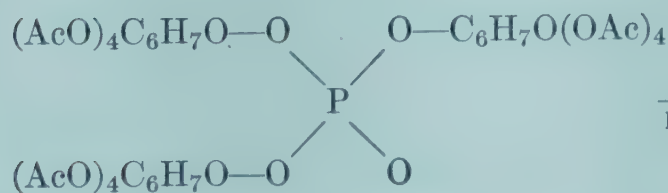
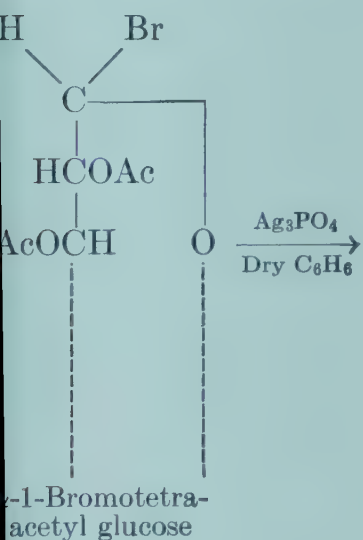
2. *Esters.* a. Acetic Acid Esters: The acetylation of the sugars has already been referred to. These compounds have been important not only for the light they throw on the structure of the sugar molecule, but because the acetyl group after serving to protect the reactive hydroxyl is easily removed by hydrolysis.

An important use is made of the acetylated sugars in the preparation of the very reactive acetylglucosyl halides which serve as starting material for many syntheses. For example, pentaacetyl glucose reacts with concentrated halogen acid in the presence of glacial acetic acid, to replace

the acetoxy group on the anomeric carbon with halogen. The halogen thus introduced is readily replaceable by various acyl and alkoxyl groups, probably through the intermediate formation of an addition product.

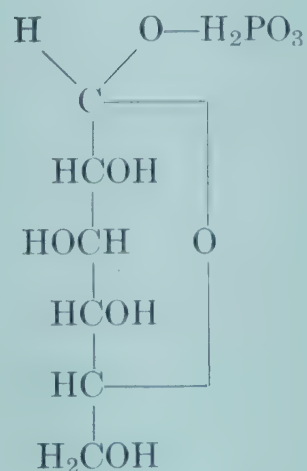


b. Phosphate Esters: Physiologically the phosphate esters are among the most important carbohydrate derivatives. Orthophosphoric acid with its three hydroxyl groups is ideally formed to link together compounds with



Tri(tetraacetyl
glucose-1-phosphate)

stepwise
hydrolysis

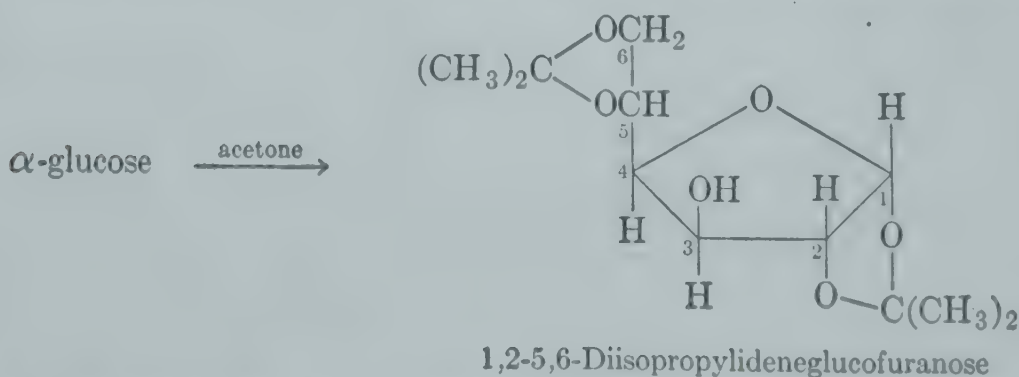


Cori ester
Glucose-1-phosphate

which it can be esterified. Thus it occurs in compounds in which it serves to link together two sugar residues, or to bind a sugar to one of several different basic compounds. An example of the chemical synthesis of a phosphoric acid ester is the one by which glucose-1-phosphate is prepared. Treatment of α -1-bromotetraacetyl glucose (the tetraacetylglucosyl bromide formulated in the previous section) with silver phosphate brings about precipitation of silver bromide and esterification of all three phosphate hydroxyl groups with three separate acetylated sugar residues. Two of the sugar residues are then removed by prolonged treatment with acid in the cold, and subsequent deacetylation yields α -D-glucose-1-phosphate (see p. 111). This is the compound which is known as the Cori ester ⁶ in honor of its discoverers.

The older structural formula for orthophosphoric acid showed one of the four oxygen atoms doubly bound to phosphorus. Since there is no distinction to be made between the various oxygen atoms in the orthophosphate ion and since, because of resonance, the double bond character is distributed among them, it seems better to omit a localized double bond.

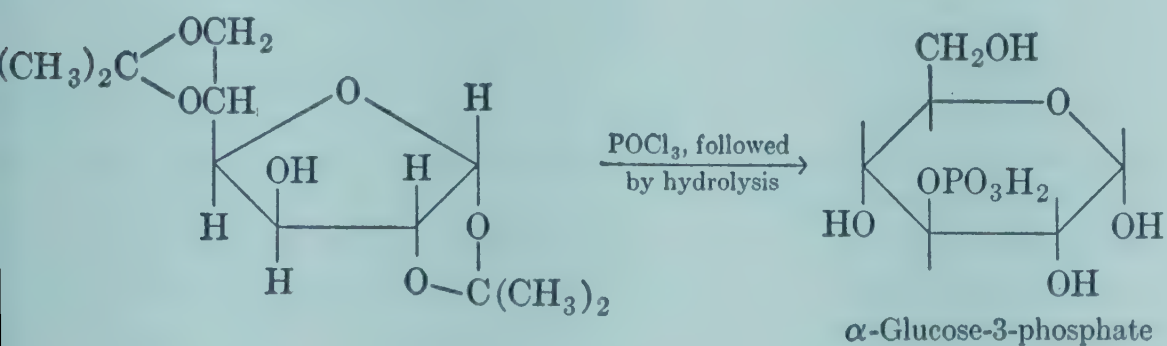
Synthesis of the phosphate derivative in which the hydroxyl group at carbon 3 of glucose is esterified, makes use of a different sort of protective grouping. It was Emil Fischer who first noted that glucose would condense with two moles of acetone to yield a diacetone derivative now known as diisopropylideneglucose. The reaction is a general one, and results from condensation of one mole of acetone with a pair of adjacent, *cis* hydroxyl groups. Since glucose yields a beautifully crystalline derivative in which it is linked to two acetone residues, it must first have undergone some sort of rearrangement. In ordinary α -glucose the hydroxyl groups on carbons 1 and 2 have the *cis* arrangement, but no other two free, adjacent ones are on the same side of the molecule. The acetone derivative proves to be 1,2-5,6-diisopropylideneglucofuranose in which, with the ring shifted to carbon 4, the hydroxyls on carbons 5 and 6 have been able to react with



⁶ Carl Cori and his wife Gerty Cori were born and educated in Czechoslovakia. Since coming to the United States in the early 1920's they have been associated with the Washington University Medical School in St. Louis. For their joint work in the field of carbohydrate metabolism they were awarded the Nobel Prize in Medicine in 1948, sharing it with Bernardo Houssay of Argentina.

acetone. Incidentally this points to the probability that a solution of glucose consists of an equilibrium mixture in which other ring forms than the pyranose are present in small amounts. When the acetone reacts selectively with the furanose form the equilibrium shifts until all of the glucose has been transformed to yield the diacetone derivative.

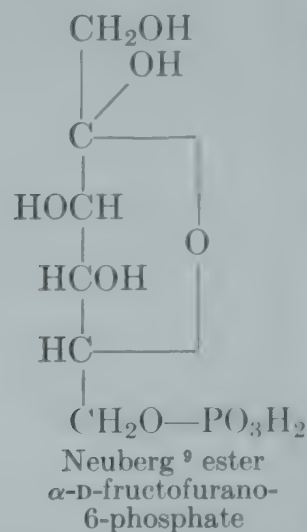
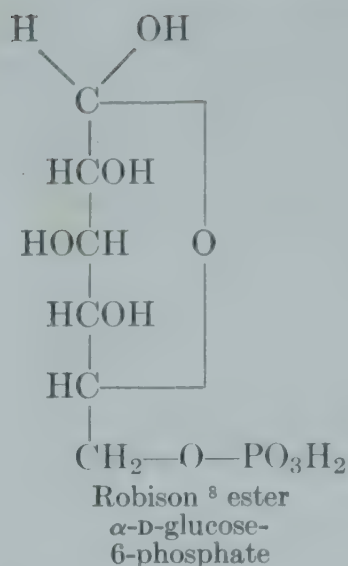
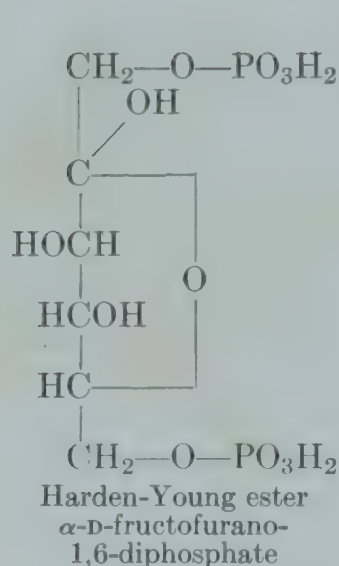
Other sugars form similar acetone derivatives, sometimes with and sometimes without a preliminary shift in the point of attachment of the oxygen bridge. The importance of the compounds so formed rests largely in the fact that the isopropylidene groups are easily removed by dilute acid hydrolysis after they have served their purpose of protecting some of the hydroxyl groups from reagents. Thus with the glucose derivative above, reaction with phosphorus oxychloride introduces a phosphate group at the only carbon which has a free hydroxyl group. Subsequent mild hydrolysis removes the blocking groups and allows the 1-5 ring to form again in the glucose-3-phosphate which results.



Similar reactions with other protected molecules have yielded various hexose and pentose phosphate esters. It was in the study of alcoholic fermentation of glucose that the importance of phosphate esters was first recognized. Later work has proved that the same compounds play an essential role in many different metabolic sequences, both in plants and in animals. The first naturally occurring esters to be identified were a hexose diphosphate and two different monophosphates. As often happens in biochemistry, these substances came to be known by the names of their discoverers. The two British pioneers in the field of alcoholic fermentation, A. Harden ⁷ and W. J. Young isolated a 1,6-fructose diphosphate which is still known as the Harden-Young ester. The formulas given on page 114 indicate the structures of the three most important hexose phosphate esters aside from the Cori ester.

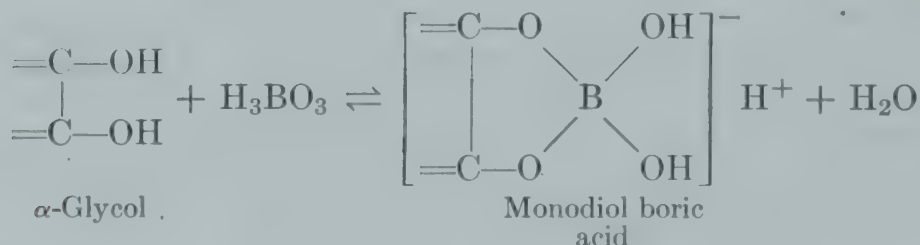
c. Boric Acid Esters: Another reaction which involves a pair of adjacent *is* hydroxyl groups is that with boric acid. In 1913 J. Böeseken of the

⁷ Sir Arthur Harden (1865–1940) was Professor at London University and Head of the Biochemistry Department at the Lister Institute of Preventive Medicine. In addition to the work of research he carried for many years the editorship of the *Biochemical Journal*. In 1929 he shared with the Swedish biochemist, Hans von Euler, the Nobel Prize in Chemistry.



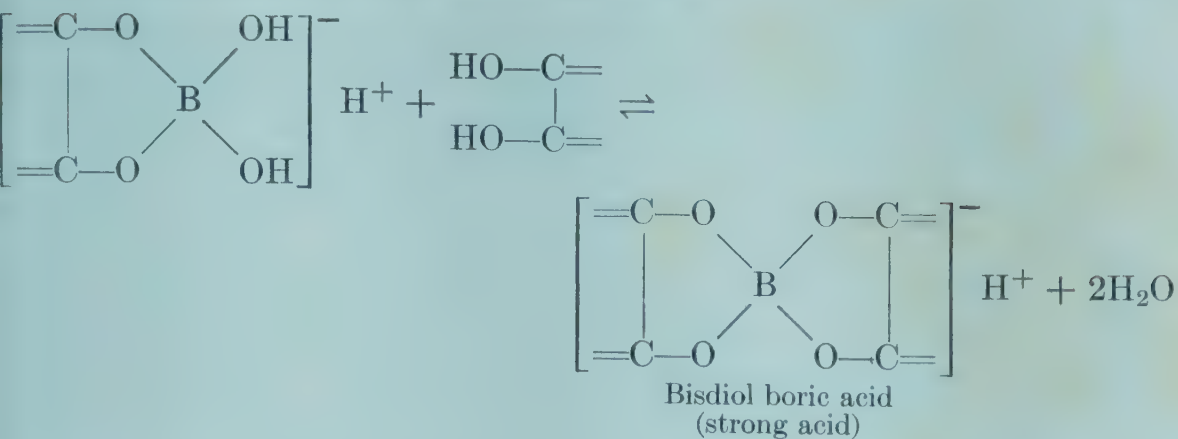
University of Delft in Holland reported that *cis*- α -glycols enhance the conductivity of the very weak boric acid, and suggested that the effect was due to the formation of complex ions. It has been found that the effect is greater when the amount of boric acid is limited, and this is now believed to depend upon the formation under those circumstances of a strong acid, bisdiol boric acid. As shown in the formulation this substance probably forms in a two-step reaction.

Since the formation of the highly ionized diglycol derivative requires that the two hydroxyl groups be in the *cis* relationship, the reaction has been useful in determination of the configuration of the sugars. For example, when α -D-glucose is added to a solution of boric acid the conductivity is greatly enhanced and then decreases gradually as mutarotation proceeds. With β -D-glucose the original conductivity is less, and increases slowly to the same equilibrium value. Böeseken interpreted these results to mean that in α -D-glucose the hydroxyl groups on carbons 1 and 2 have the *cis* arrangement, whereas in the β -form they are *trans*.

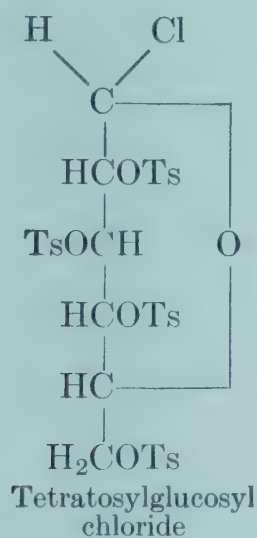


⁸ Professor Robert Robison (1883–) has been at the Lister Institute in London since 1913 and is now Head of its Department of Biochemistry and Professor of Biochemistry at London University.

⁹ Carl Neuberg (1877–) has been for many years an outstanding figure among those concerned with enzyme chemistry in general and with alcoholic fermentation in particular. In 1938 he became Professor Emeritus at the University of Berlin after a long and fruitful career there. Between 1941 and 1948 he was Research Professor of Chemistry at New York University, again becoming Emeritus in 1948. Since then he has been carrying on his research at the Polytechnic Institute in Brooklyn.

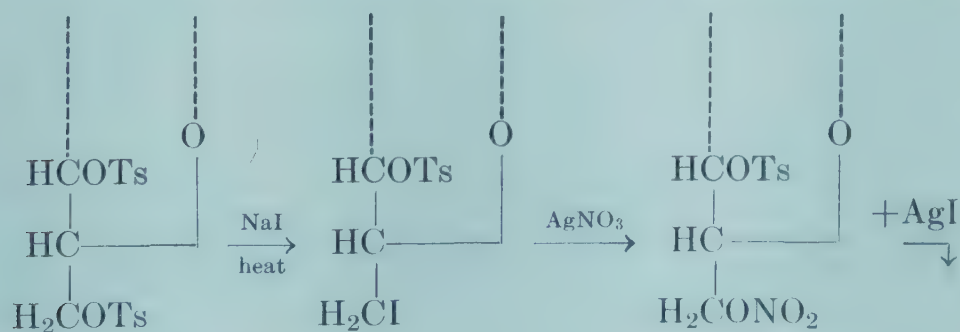


d. Tosyl Esters: The acid chlorides of the aryl sulfonic acids react with glucose as do other acid chlorides to form the corresponding esters. Thus *p*-toluenesulfonyl chloride in pyridine solution reacts with glucose to form a derivative in which four hydroxyl groups have been esterified, while the hydroxyl on carbon 1 has been replaced by chlorine. The compound is



called tetratosylglucosyl chloride, and the symbol Ts is used to represent the $\text{CH}_3\text{—C}_6\text{H}_4\text{—SO}_2\text{—}$ group.

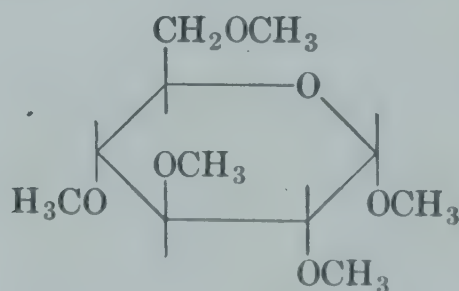
One of the chief uses of the tosyl esters is in testing for the presence of primary alcoholic groups. When a polytosyl ester is treated with sodium iodide in acetone solution, the salt reacts preferentially with a tosyl group attached to a primary alcoholic carbon. Iodine replaces the tosyl residue and sodium *p*-toluenesulfonate is set free. On treatment of the sugar



iodide with silver nitrate, the insoluble silver iodide is precipitated and may be determined quantitatively, thus measuring the number of primary alcoholic groups in the original compound.

3. *Ethers*. a. Methyl Ethers: Brief reference has already been made to the importance of the methylation procedures by which sugar ethers may be prepared. The first successful method was that of the St. Andrews chemists, Purdie and Irvine,¹⁰ making use of a mixture of methyl iodide and silver oxide. The reaction of alcoholic hydroxyl groups with this reagent was discovered accidentally when Purdie attempted to esterify lactic acid by treating it with methyl iodide in the presence of silver oxide. The properties of the ester which formed proved to differ slightly from those of a supposedly identical ester prepared by Emil Fischer using his methyl alcohol-hydrochloric acid reagent. Search for the cause of the discrepancies led to the isolation of a small amount of a substance which proved to be the dimethyl derivative, $\text{CH}_3\text{—CH(OC}_2\text{H}_5\text{)—COOC}_2\text{H}_5$. The importance of this formation of an ether linkage led to an elaboration of the method for the preparation of sugar ethers.

The procedure which is now commonly used for methylation was developed by Haworth. The reagent is dimethyl sulfate, used with 30 per cent sodium hydroxide. It is very much cheaper than the Purdie reagent and does not have the disadvantage of including an oxidizing agent (Ag_2O) which attacks the reducing groups if it is used with unsubstituted sugars. Complete methylation with either reagent usually requires several repetitions of the procedure. With methyl iodide and silver oxide the complete methylation of glucose may require six or more treatments with the reagents. The final product is a methyl tetramethylglucoside.



Tetramethyl- α -methylglucopyranoside

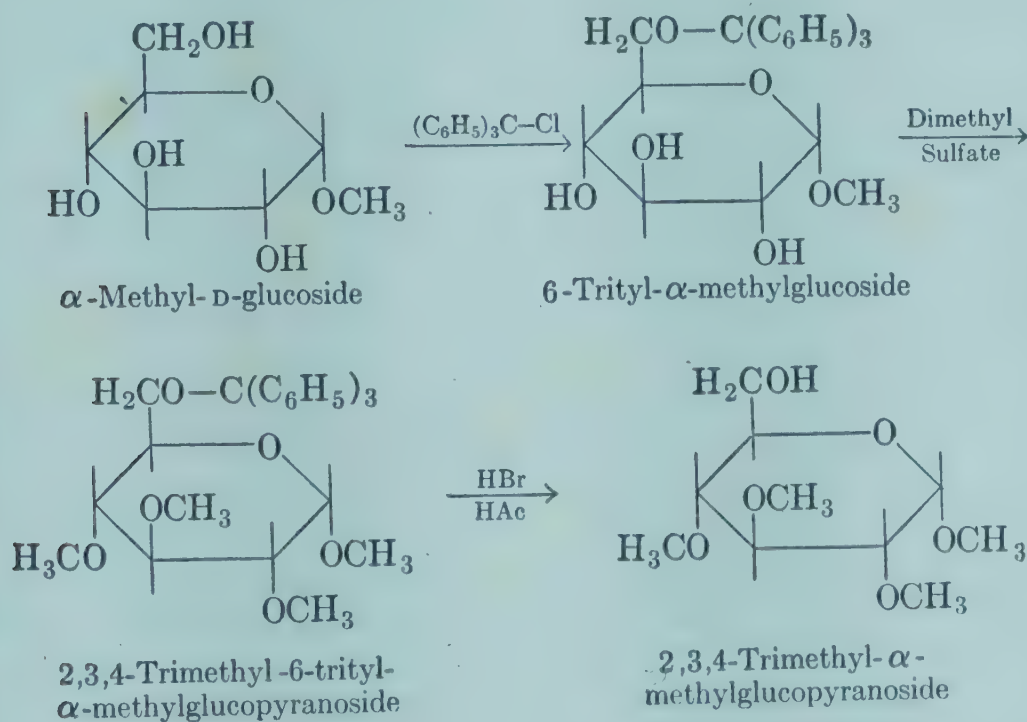
It should be noted that while this compound contains five methoxyl groups, the group on carbon 1 is not held in a stable ether linkage. If the penta-

¹⁰ Thomas Purdie (1843-1916) preceded Irvine (1877-1952) in the Professorship of Chemistry at the University of St. Andrews in Scotland. James Colquhoun Irvine (later Sir James), though burdened with heavy administrative duties after 1921 when he became Principal of the University, yet carried on an active research program until about 1938. His pioneer work on carbohydrate structure was a fundamental contribution to that complex subject.

methyl compound is hydrolyzed the glucosidic methyl group on carbon 1 is removed and there results a tetramethyl glucose.

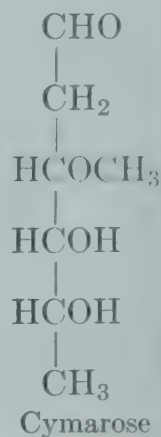
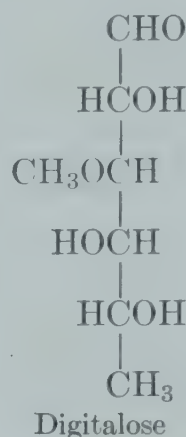
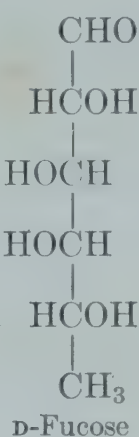
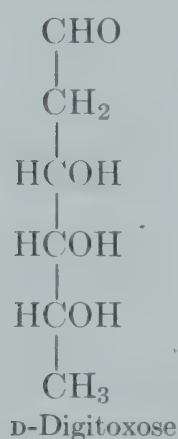
The partially methylated sugars have been of great importance as reference compounds in determinations of the structure of oligo- or polyaccharides. They are usually prepared by blocking some of the hydroxyl groups before methylating and removing the protecting groups after methylation is complete. By a variety of such procedures there have been prepared several partially methylated derivatives of the common hexose and pentose sugars. We shall see later how these compounds are used in formulation of the structures of the more complex carbohydrates.

b. Triphenylmethyl Ethers: The ethers formed by interaction between triphenylmethyl chloride and alcoholic groups are known as "trityl" ethers. The reagent attacks any primary hydroxyl group much more readily than a secondary group, and under proper conditions will block only the primary position. For example, α -methylglucoside reacts in pyridine solution to form a 6-trityl derivative which can then be methylated or acetylated at the other three hydroxyl groups. Although the trityl group is held in an ether linkage it can be removed by hydrolysis with a mixture of acetic and hydrobromic acids without splitting the glucosidic bond.



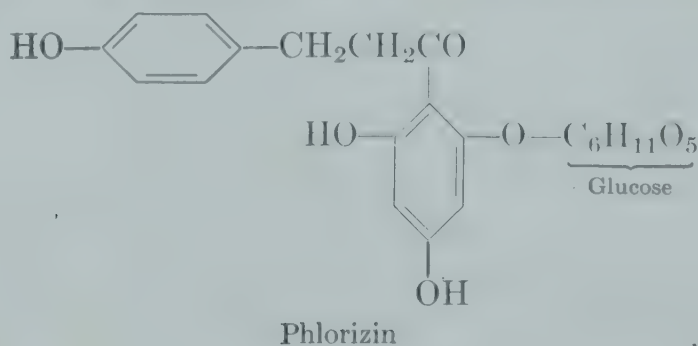
4. *Naturally Occurring Glycosides.* There occur naturally in the plant world a large number of compounds which on hydrolysis yield one or more sugar molecules, and one or more molecules of some other type. Many of these were known and had been used in commerce long before Fischer prepared his methylglucosides. They were originally known as glucosides, but since the sugar moiety is not always glucose, the modern class name

is *glycoside*, with glucoside used only for those compounds which yield glucose when hydrolyzed. The nonsugar part of these compounds is known as the *aglycone* or *aglucone*. It may be a simple compound like methyl alcohol, which is the aglucone of the methylglucosides, or it may be a much more complex substance. The sugar components are also various, and include besides simple hexose and pentose residues a number of substances closely related to the sugars. Below are given the simple straight chain formulas of several sugar derivatives which have been found in natural products.



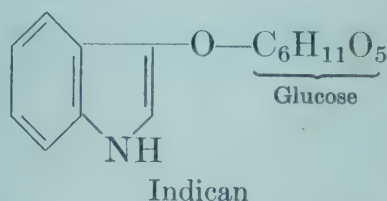
The aglycone portions of plant glycosides are often toxic substances, and it has been suggested that their condensation with carbohydrates may be a detoxication mechanism of the plant. On the other hand it may be that they protect the plant in another way. Wherever a glycoside is found in a plant there is also, presumably in neighboring cells, an enzyme or enzymes capable of bringing about its hydrolysis. On this basis it has been suggested that a phenolglycoside, for example, may serve to hold the phenol in a nontoxic form. If a part of the plant is injured the glycoside and the enzyme come into contact and the antiseptic phenol is set free at the point of injury. This may well be the function also of those glycosides, such as amygdalin, which release hydrogen cyanide on hydrolysis.

Among the phenolic glycosides phlorizin, found in the bark of plum and apple trees, has been widely used in biochemical studies. When injected into an animal it induces an excretion of sugar which simulates diabetes, and this was for many years one of the two standard ways of inducing experimental "diabetes" in animals. On hydrolysis of the glucoside, glucose

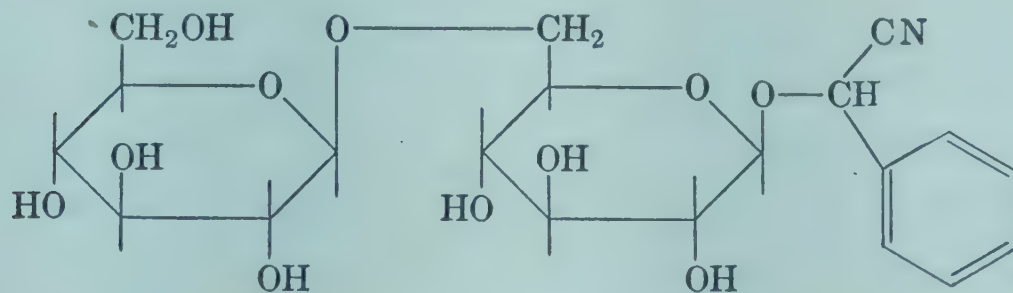


is set free, while the aglucone is a polyhydroxy aromatic ketone phloretin (2,4,6-trihydroxy β -[4-hydroxyphenyl]propiophenone).

Many of the plant colors are attributable to the presence of glycosides. One type of aglycone (the hydroxyflavones) introduces yellow and brown tints, while another (the anthocyanins) is associated with reds and blues. The familiar dye, indigo blue, is prepared by oxidation of the hydroxy-indole, indoxyl, which is the aglucone of the plant glucoside, indican.



Amygdalin is a glucoside of great historical interest, since it was one of the earliest to be formulated, and also because the first recognition of those potent biological catalysts the enzymes came about during a study of its properties. It is found in the seeds of the bitter almond, and yields on complete hydrolysis two molecules of glucose and one each of benzaldehyde and hydrogen cyanide. More careful enzymic hydrolysis as well as synthesis have now established the fact that the sugar occurs as a disaccharide, gentiobiose, while the aglycone is mandelonitrile.



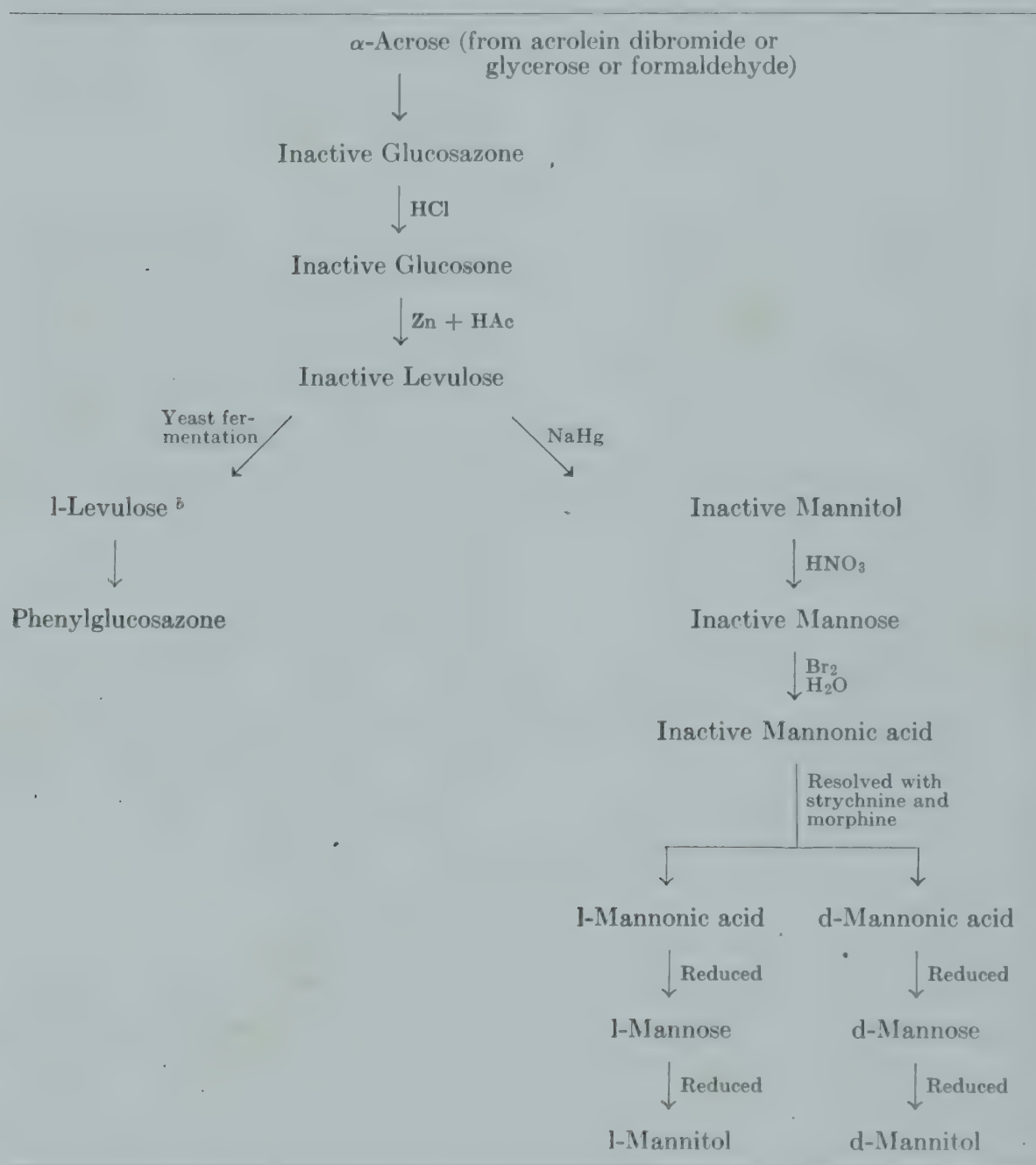
Amygdalin

SYNTHESIS AND DEGRADATION OF THE SIMPLE SUGARS

Synthesis from Noncarbohydrates. The unique example of carbohydrate synthesis from inorganic molecules is photosynthesis by green plants. The chemistry of this process has proved to be so complex that it cannot profitably be considered at this point, but will be taken up in Chapter 15.

The nearest approach to a laboratory synthesis of carbohydrate was carried out by Emil Fischer. He found that three different simple substances could be made to yield reducing sugars. Whether he treated glycerose with bromine and sodium carbonate, or allowed mild alkali to act upon acrolein dibromide or upon formaldehyde, he obtained optically inactive reducing mixtures which were enough alike to be designated by the single name of "acrose." From several of these solutions Fischer

isolated inactive glucose as the phenylosazone. Using this racemic mixture as his new starting material, he carried out by the usual methods of sugar chemistry an outstanding series of syntheses of sugars and sugar derivatives which established the relationships between a number of the simple sugars. Some of the transformations which he effected are shown in Table 4-II

TABLE 4-II. FISCHER'S SYNTHESIS OF SUGARS ^a

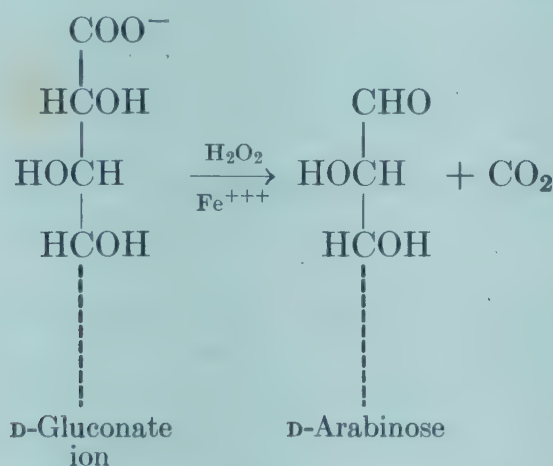
^a From E. Fischer, "The Synthesis of Mannose and Levulose," *Ber.*, 23:370, 1890.

^b The d- and l- in this table are used as Fischer used them to indicate the actual rotation of the compounds.

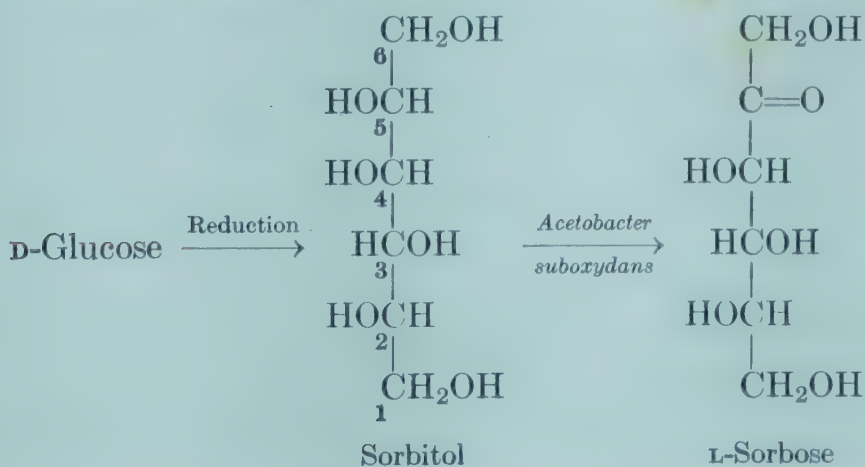
adapted from a similar chart in Fischer's paper. It would be an excellent exercise in carbohydrate chemistry to make sure that each step in the procedures is clearly understood!

Lengthening the Aldose Chain. Reference has already been made to the Kiliani synthesis by which it is possible to synthesize from one aldose the next higher member of the series (p. 86). Theoretically it would be possible to prepare all the aldohexoses by repeated cyanohydrin syntheses from the two glyceroses. Not all of these reactions have actually been carried out but many of them have, and further, hexose molecules have been lengthened to yield sugars with as many as ten carbons in a straight chain.

Shortening the Aldose Chain. One of the best methods for shortening sugar chains depends upon the fact that a salt of an aldonic acid can be oxidized by hydrogen peroxide in the presence of ferric ion. Carbon dioxide is liberated yielding the next lower sugar of the series. Thus D-gluconic acid gives rise to D-arabinose.

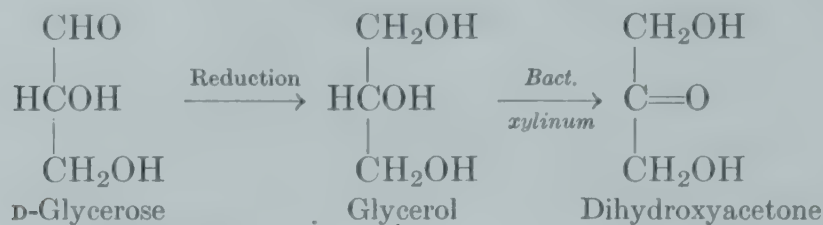


Bacterial Syntheses. Green plants are important because they furnish many carbohydrates of commercial importance, and certain bacteria are now valuable because carbohydrates and carbohydrate derivatives are by-products of their metabolic activities. Various strains of *Acetobacter*, for example, ferment sugar alcohols with formation of ketoses. Thus L-sorbose, an isomer of fructose, is prepared commercially from D-glucose by first reducing the sugar to the corresponding alcohol, and then fermenting the alcohol by bacteria. Note that the sorbitol formula is written with carbon



6 at the top. The ketose which is formed in this fermentation finds a wide use in the preparation of vitamin C.

A similar pair of reactions serves to transform glycerose to the ketotriose, dihydroxyacetone, thus again achieving the transformation of an aldose to a corresponding ketose.

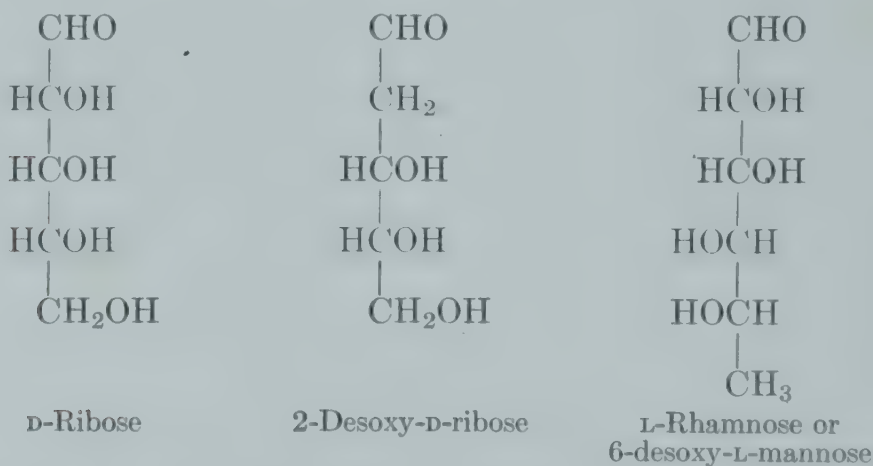


The field of bacterial synthesis is a wide one and is being exploited in the preparation of other substances than sugars. For a survey of the reactions which can be brought about in this way the student should consult a text devoted to bacterial metabolism.

Compounds Related to the Monoses

DESOXY SUGARS

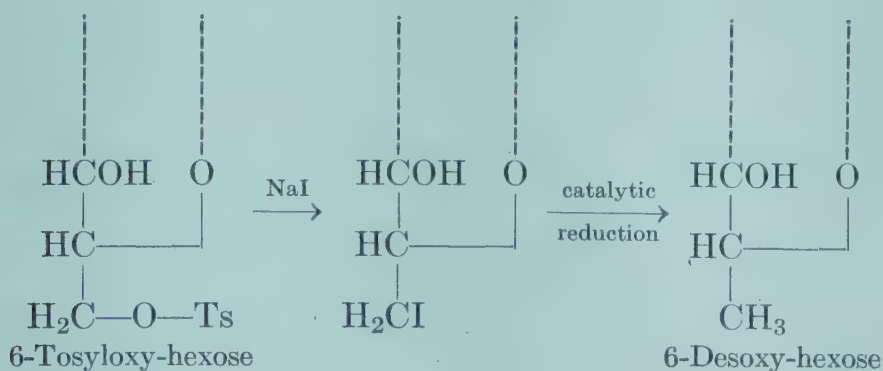
Reference has already been made in connection with the natural glycosides to compounds which differ from the simple sugars only in lacking one or two oxygen atoms. These substances, known as desoxy (or deoxy) sugars, occur in several compounds of great physiological importance. One of the nucleic acids which are found in all living cells contains a desoxy derivative of the aldopentose, ribose, while a 6-desoxy hexose, L-rhamnose, is a constituent of many glycosides and polysaccharides.



Compounds like rhamnose, having a terminal methyl group, are also named as methyl pentoses, rhamnose itself being methyl-L-lyxose.

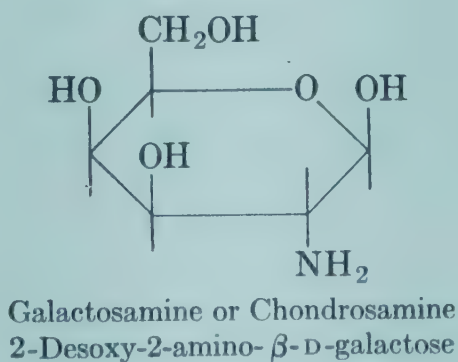
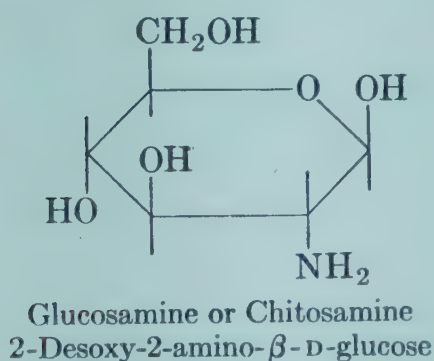
The 6-desoxy sugars are readily prepared from the corresponding hexoses through the tosyl derivatives. It will be remembered that a tosyl residue

on a primary carbon can be replaced by iodine. Reduction of such an iodo compound converts it to the desoxy sugar.



AMINO SUGARS

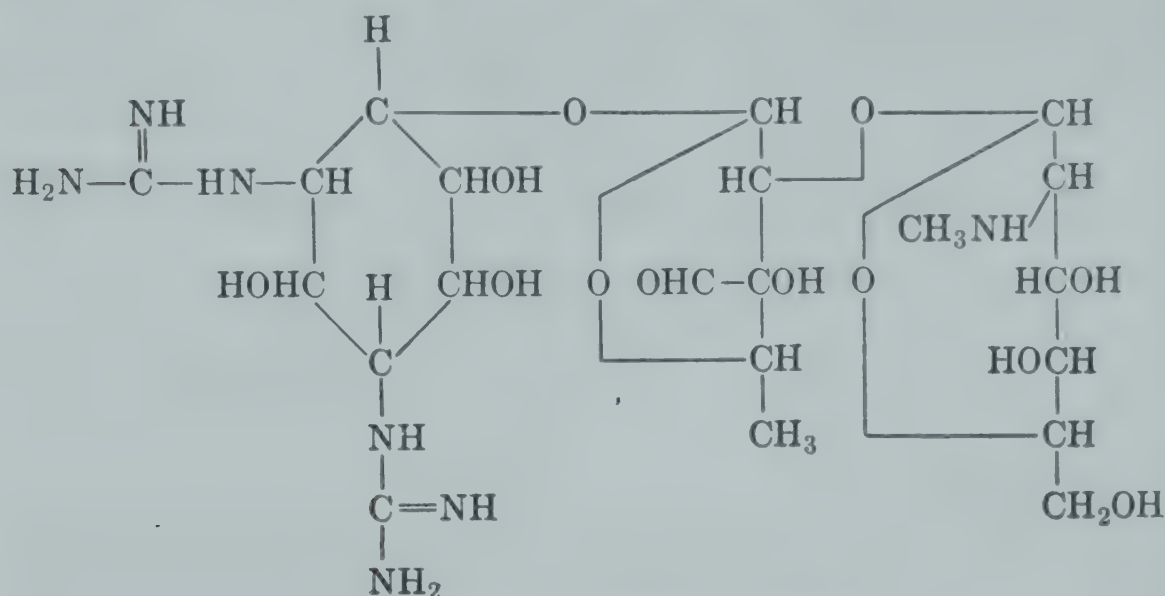
The hexosamines are examples of the group of amino sugars, derivatives which have an amino group in place of one alcoholic hydroxyl. The two important natural representatives of the group are *chitosamine*, or D-glucosamine and *chondrosamine* or D-galactosamine. The former is obtained by hydrolysis of chitin, the skeletal polysaccharide which makes up most of the horny parts of crustacea and insects. Chondrosamine is one of the building blocks of another polysaccharide, chondroitin sulfuric acid, which is found in cartilage and tendons. Recent work has shown that the amino sugars have the configurations indicated in the projection formulas.



Since with most amino polysaccharides, hydrolysis liberates equimolecular amounts of amino sugar and of acetic acid, it is probable that in the polymerized form the amino groups are all acetylated.

Special interest attaches to the amino sugars because of their presence both in certain bacterial polysaccharides, and in the antibiotic, streptomycin. The significance of the bacterial polysaccharides will be discussed in a later section (p. 148). Streptomycin is also a bacterial product, elaborated by the soil organism, *Streptomyces griseus*. In structure it is a di-glycoside in which a methylated amino sugar is united through a

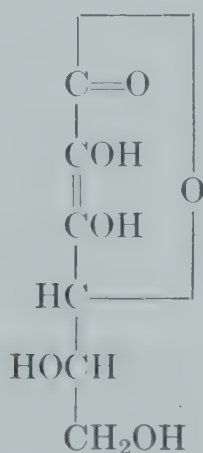
glycosidic link to a modified, branched chain hexose, which is in turn glycosidically linked to a substituted cyclic alcohol.



Streptomycin (tentative formula)

ASCORBIC ACIDS

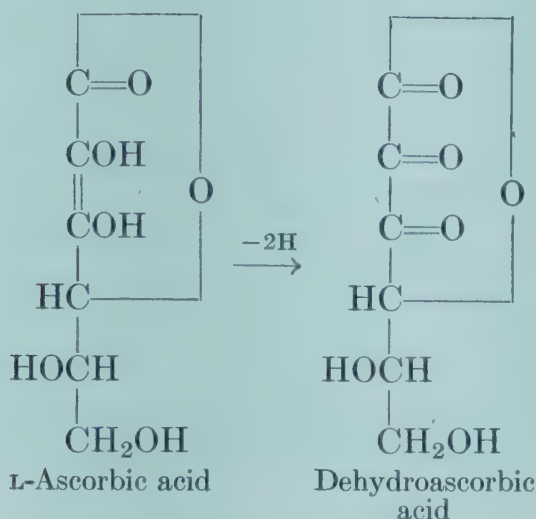
Closely related to the sugars are the unsaturated lactones known as ascorbic acids, of which vitamin C is the most important. It is found in many animal tissues and also in plentiful supply in citrus fruits and green vegetables. It was first isolated by Szent Györgyi¹¹ in 1928 and because of its acidity and its empirical formula ($C_6H_8O_6$) was named hexuronic acid. It has been shown to have the structure of a lactone of an enediol hexonic acid.



Ascorbic acid

¹¹ One of the most colorful figures in modern biochemistry is that of Albert von Szent Györgyi (1893-). Until 1947 his work was done in Hungary and it was at the University of Szeged that he isolated vitamin C. He is an unconventional thinker whose stimulating ideas are always expressed with wit and charm. In 1937 he was awarded the Nobel Prize in Medicine for his contributions to theories of biological oxidation. He is at present working at the Institute for Muscle Research at Woods Hole, Massachusetts.

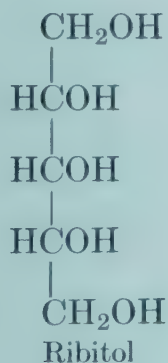
Although it behaves like a typical acid, titrating with bases and decomposing carbonates, the acidity of ascorbic acid resides in the hydroxyl groups attached to the doubly linked carbons. This is shown by the fact that after oxidation, which is readily brought about by iodine, the compound is no longer acidic.



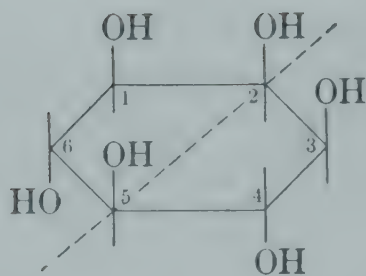
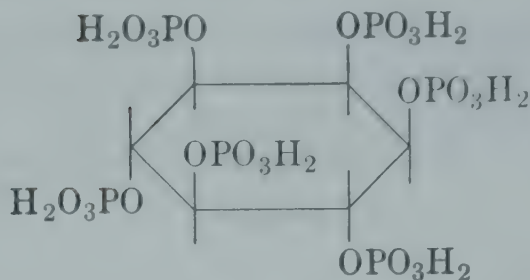
Compounds similar in structure to L-ascorbic acid show varying degrees of antiscorbutic activity, but this proves to be true only of those forms in which the lactone ring is on the right. This is one of many examples of the fact that living cells often distinguish between stereoisomers and show a specific preference for one of two forms.

CYCLOHEXANOLS

Although several straight chain polyhydroxy alcohols occur naturally, none are of biological interest except the one related to ribose. This is the compound ribitol which is part of the molecule of riboflavin or vitamin B₂.



The cyclic hexahydric alcohols or *inositols*, on the other hand, appear in many compounds of biological importance. In the phytin which is found in many plant seeds, *meso*-inositol occurs as the calcium magnesium salt of the hexaphosphate ester, phytic acid.

*meso*-Inositol

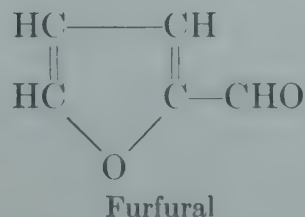
Phytic acid

As its name indicates, *meso*-inositol is optically inactive, in spite of having an apparently unsymmetrical arrangement of hydroxyl groups above and below the plane of the ring. Its plane of symmetry is indicated in the formula by a dotted line. It is the most abundant of all the inositols, occurring both free and in combination in plant and animal tissues. One of the two yeast growth factors, Bios I, is *meso*-inositol; a nitrogenous derivative is one of the building blocks in streptomycin, and the alcohol itself makes up about 7 per cent of a complex phospholipid found in brain tissue.

Analytical Reactions of the Sugars

COLOR TESTS

Molisch Reaction. The most general test for the sugars depends upon a reaction with α -naphthol. When any carbohydrate or any compound containing a carbohydrate residue, is mixed with an alcoholic solution of α -naphthol and then treated carefully with concentrated sulfuric acid, a reddish-violet color appears at the zone of contact between the two layers. This is due to formation of a condensation product of the naphthol with either furfural or a furfural derivative. In the presence of very strong acid any carbohydrate yields the required cyclic compound.



Seliwanoff Reaction. This reaction serves as a test for the presence of a ketose, and depends upon the fact that ketoses yield furfural derivatives under conditions of acidity so mild that the more stable aldoses are unaffected. The reagent contains resorcinol in dilute hydrochloric acid solution, and when it is added to a solution of a ketose the resorcinol forms a clear bright red condensation product with the furfural from the sugar. Clearly such a test has meaning only if the prescribed conditions are

rigidly adhered to. Excessively prolonged heating yields a positive test with sucrose, glucose, and maltose, with the first sugar because hydrolysis frees the ketose, with the others because long exposure to acid eventually decomposes even aldoses. For the same reason the concentration of the acid must be carefully controlled.

Pentose and Uronic Acid Tests. The pentoses and uronic acids are like the ketoses in being more sensitive to acid than are the aldoses. They consequently form furfural derivatives under comparatively mild conditions.

Tauber's Test: In glacial acetic acid both pentoses and uronic acids react with benzidine to produce a cherry red color which is easily distinguishable from the yellowish brown which develops in the presence of an aldohexose.

Bial's Test: The sugar solution is treated with a reagent which contains orcinol, ferric chloride, and concentrated hydrochloric acid. A green condensation product which first forms in solution and may later precipitate is a positive test for a pentose.

Naphthoresorcinol Test: In the presence of strong mineral acid, the uronic acids give rise to a purple color with naphthoresorcinol.

REDUCTION TESTS

It has already been pointed out that the reaction between glucose or fructose and such an oxidizing agent as Fehling's solution is not a stoichiometric one. As should be evident from that discussion, the extent of the oxidation depends upon the concentration of the base and upon the temperature and time of heating. In spite of these variations, oxidizing solutions are used successfully to estimate reducing sugars quantitatively. This requires that the conditions of the reaction be meticulously standardized and that successive determinations be carried out under identical conditions. Then by comparing the extent of reduction brought about by an unknown solution with that caused by a known quantity of glucose, it becomes possible to calculate the quantity of reducing sugar in the experimental sample, even though the actual products are not known. Below are listed some of the more important oxidizing solutions used in biochemical work.

Fehling's Solution. This was one of the earliest alkaline copper reagents. It was used for quantitative estimation of relatively large amounts of reducing sugar by filtering and weighing the cuprous oxide which precipitated under specified conditions.

Benedict's Methods. Stanley Benedict of Cornell University Medical College described several different modifications of the alkaline copper reagent, designed to make it possible to test for glucose in the presence of such other reducing substances as are found in biological fluids. His methods are adapted to the estimation of very small quantities of reducing sugar and are largely colorimetric. The cuprous oxide is first precipitated by the sugar under rigidly controlled conditions. There is then added a

so-called "color reagent" containing a colorless complex phosphomolybdate which can be reduced to a clear blue derivative. The cuprous oxide brings about such a reduction, and the depth of the blue color is a measure of the amount of oxide present, and this in turn depends upon the original quantity of reducing sugar. By comparison of the color in the experimental tube with that which develops in a standard tube containing a known quantity of glucose, it is possible to calculate the glucose concentration of the unknown solution.

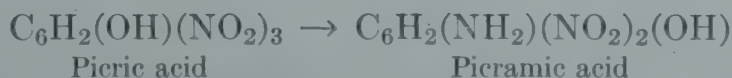
Schaffer-Hartmann Method. In this method an alkaline copper reagent is used, but the final estimation is titrimetric. After the preliminary reduction of cupric ion by the sugar, the cuprous oxide which has formed is reoxidized by free iodine. The conditions are such that the same accurately



standardized amount of iodine is added to experimental tubes and to a tube in which a known quantity of glucose has reacted to precipitate cuprous oxide. Comparison of the volumes of iodine solution required in the two cases makes it possible to calculate the concentration of sugar in the unknown solution. The reagent may be made up in either a macro or a micro modification, and can be used for estimation of amounts of glucose as small as 0.1 mg. or as large as 200 mg.

Hagedorn-Jensen Method. This micro method, which estimates as little as 0.002 mg. of glucose, depends upon the reduction of ferricyanide to ferrocyanide ion. The reaction is carried out in such a way that the ferrocyanide which forms precipitates as a mixed salt, $\text{K}_2\text{Zn}_3(\text{Fe}[\text{CN}]_6)_2$. With this out of the way, the residual ferricyanide is estimated by a titrimetric procedure and the glucose concentration calculated from the titration figures.

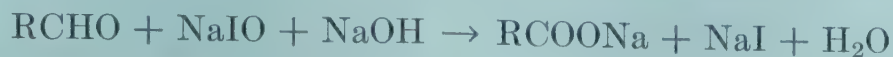
Picric Reagent. All biological fluids contain more or less colloidal protein which must be removed before any sort of quantitative procedure can be carried out. Picric acid is one of the reagents which precipitate proteins and it has the added property of being reduced by glucose in alkaline solution to yield the mahogany red salt of picramic acid. After addition of picric acid to blood, for example, the precipitated proteins are filtered



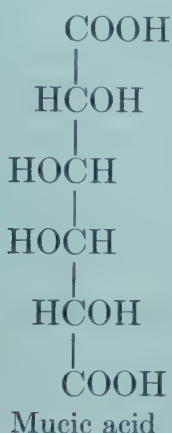
and the red color of the filtrate is a measure of the quantity of glucose present in the blood.

Nylander's Solution. This is an alkaline bismuth reagent which precipitates metallic bismuth when it reacts with reducing sugars. Under proper conditions it may be used as a delicate qualitative test for the presence of such sugars.

Aldose Titration. An oxidation in which aldoses are oxidized quantitatively to aldonic acids is brought about by alkaline hypoiodite. By rigid control of the alkalinity and the temperature, it is possible to estimate the concentration of aldoses in the presence of ketose sugars.

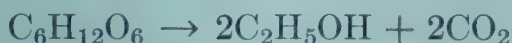


Mucic Acid Test. By oxidation with concentrated nitric acid it is possible to distinguish galactose and lactose from other sugars. The test depends upon the fact that mucic acid (*galacto-saccharic acid*) is the only one of the saccharic acids which is sufficiently insoluble in water to crystallize from concentrated solution. Since lactose yields a molecule of galactose on hydrolysis it also gives a positive mucic acid test.



FERMENTATION TESTS

Conversion of sugar to alcohol in the presence of yeast takes place according to the equation



Among the hexoses only D-glucose, D-mannose, and D-fructose are readily fermented by bread yeast. D-Galactose ferments with difficulty and pentoses do not ferment at all. These facts have long been used to distinguish between the various sugars, either by noting whether or not carbon dioxide was evolved in the presence of yeast, or by testing for the presence of alcohol after incubating an unknown sugar with yeast.

Of late years this method has been greatly elaborated. The fermentative requirements of many different microorganisms have been determined and prove to be rather specific. For example, *B. megatherium* can use L-arabinose but not the D-form; it ferments D-glucose and D-ribose but not D-galactose or L-ribose. With some sugars the products are acidic, for there are other fermentative reactions than the one noted above, while other sugars yield carbon dioxide. Since data of this sort are now available for a long list of microorganisms, it is possible to use selective fermentation tests both for qualitative and quantitative estimation of sugar mixtures.

For example, galactose can be determined in the presence of mannose, glucose, fructose, xylose, arabinose, and glucuronic acid by testing the mixture with two different yeasts, one of which (*Saccharomyces carlsbergensis*) ferments galactose while the other (*S. bayanus*) does not. Since both ferment the other three hexoses, while neither attacks the pentoses or the uronic acid, evolution of a larger amount of carbon dioxide by *S. carlsbergensis* is a positive test for the presence of galactose. Such methods find frequent application in dealing with mixtures obtained by hydrolysis of plant gums. They are also sometimes used in reverse to identify a microorganism.

The Oligosaccharides

There have been found in plant and animal tissues a number of compounds of lower molecular weights than the polysaccharides, which yield on complete acid hydrolysis either simple sugars or closely related compounds. The empirical formulas of these substances indicate that the monoses are linked by elimination of water between two simple carbohydrate molecules, so that a general formula might be $x(C_nH_{2n}O_n) - (x - 1)H_2O$. These small condensation products are known as oligosaccharides (Gr. *oligos*, a few), though the group is not rigidly defined. At one time it included compounds made up of two to six sugar residues. More recently it has been extended to take in compounds containing as many as nine monose units. Between these and the true polysaccharides there is an enormous gap since the latter are made up of at least several hundred monose residues and may include several thousand.

It is difficult to avoid confusion between the names of the monosaccharides and the oligosaccharides. By convention a diose is a monosaccharide having two carbons, while a disaccharide is an oligosaccharide made up of two monose residues. Yet in naming specific disaccharides the suffix "biose" is used, as for example in the name "melibiose" which is a disaccharide found in plant exudates. While glyceraldehyde is classified as a triose, the same combining form used in the name of a specific sugar indicates that it is a trisaccharide. For example, the melitriose molecule contains a glucose residue linked to one of galactose and one of fructose. The tetroses, such as erythrose, are distinguished from the tetrasaccharides by calling the latter *tetraoses*, and in naming the larger oligosaccharides the "a" is similarly retained. Thus the pentaoses and the hexaoses indicate by their rather awkward names that they are oligosaccharides containing five or six monose residues.

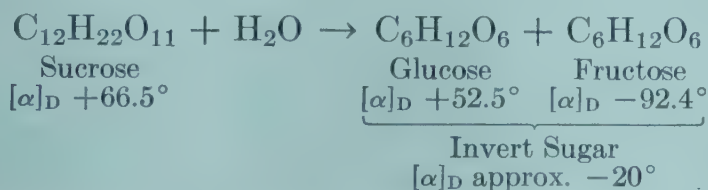
The oligosaccharides include a wide variety of natural products. Some of the compounds are nonreducing, showing that all the potential reducing groups are involved in linkages. Others have one such group free. Some of the compounds yield on hydrolysis only one type of monosaccharide while others contain two or more different sugar residues. All are hy-

hydrolyzed by one or another of the enzymes which attack glycosidic linkages, and they are therefore essentially glycosides in which the "aglycone" is also a sugar or a sugar derivative.

The chief reactions of the oligosaccharides are those of their constituent monosaccharides. Those compounds which contain a free potential reducing group react as glucose does with carbonyl reagents, forming corresponding oxidation and reduction products, oximes, osazones, etc. The hydroxyl groups react to form similar derivatives, and the glycosidic bonds are broken by the same procedures which bring about cleavage of the simple glycosides. The only matter which need be specially considered in connection with the oligosaccharides is the type of linkage which holds the sugar residues together.

NONREDUCING OLIGOSACCHARIDES

Sucrose ($C_{12}H_{22}O_{11}$) is a disaccharide and the most important non-reducing sugar. Having no free potential reducing group, it neither mutarotates in solution nor reacts with carbonyl reagents. It is hydrolyzed by acids or enzymes to a mixture of equimolecular amounts of glucose and fructose. This transformation is known as *inversion* of sucrose since the optical rotation changes from dextro to levo in the process. The specific rotation of the invert sugar is an average of that of the two components,

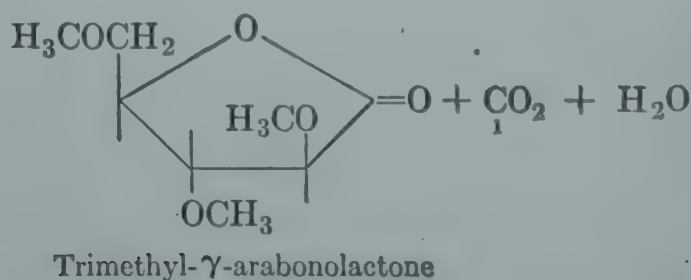
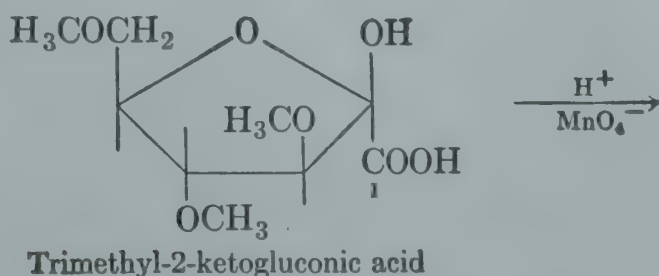
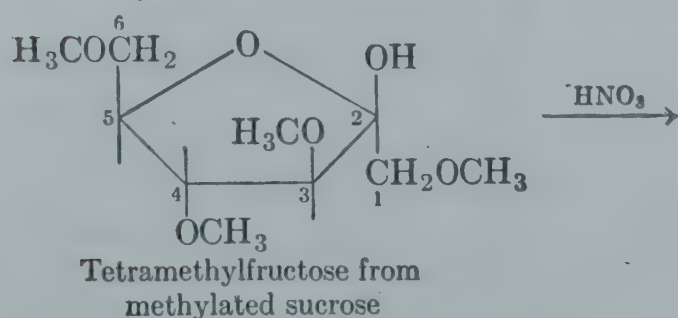


allowing for the fact that a solution which contained 1 g. of sucrose per ml. would change to one containing approximately 0.5 g. each of the hexoses.

Since sucrose is nonreducing, the two monose residues must be linked from carbon 1 of glucose to carbon 2 of fructose. The only question then concerns the positions of the two rings in the disaccharide. The products isolated after inversion are D-glucopyranose and D-fructopyranose, but methylation studies show that this does not necessarily indicate that both rings were six membered in the original sugar.

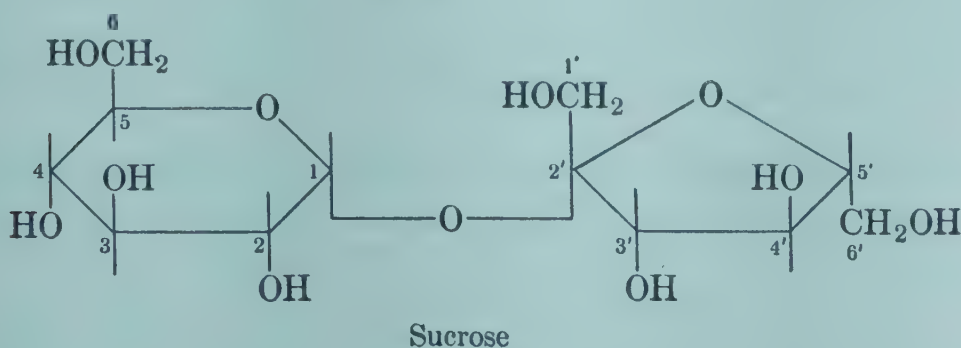
Purdie and Irvine were the first to methylate sucrose completely. They obtained an octamethyl derivative in which every free hydroxyl was blocked, and the rings therefore prevented from shifting. Hydrolysis of this product yielded the familiar 2,3,4,6-tetramethylglucose, and a tetramethyl derivative of fructose. The first of these indicated that the glucose was present in sucrose in the usual pyranose form. Since separation of the two tetramethyl compounds proved difficult, the problem of the structure of the substituted fructose was attacked indirectly. From the observed

specific rotation of the mixture of methylated sugars and the known rotation of tetramethylglucose, it was possible to calculate the rotation of the methylated fructose to be about $[\alpha]_D + 30^\circ$. This is far from the rotation of the known tetramethylfructopyranose ($[\alpha]_D - 121^\circ$), and the fructose component in sucrose must therefore be differently constituted. The structure of the tetramethylfructose from cane sugar was finally established by oxidations which transformed it in several steps to a known trimethyl- γ -arabonolactone. It should be recalled here that although the stable ring forms in the free sugars are six membered, the stable lactones of the sugar acids are the five-membered γ -rings. The presence of a five-membered ring in the lactone derived from the tetramethylfructose could only mean that the original fructose derivative had been 1,3,4,6-tetramethylfructose and that the ring in the fructose part of cane sugar is a furanose ring. The oxidation of the methylated fructose is formulated as follows:



The presence of the methylated primary alcohol group at position 5 in the arabonolactone shows that this, which was position 6 in fructose, must have been free to be methylated in the disaccharide, and could therefore not have been involved in ring formation. The furanose form of fructose which presumably is set free at the moment of hydrolysis is unstable, and rearranges immediately to the more stable pyranose form.

Before the complete formula for sucrose can be written, the configurations about the anomeric carbons must be known. When sucrose hydrolyzes under the influence of maltase the glucose which is liberated mutarotates in a downward direction. This indicates that the glucose has the α -arrangement at carbon 1. The fact that sucrose is also hydrolyzed by yeast invertase which is specific for β -fructofuranosides makes it probable that the fructose moiety has the β -configuration. Thus sucrose is α -D-glucopyranosido- β -D-fructofuranoside, and the linkage is from carbon 1 of glucose to carbon 2' of fructose.



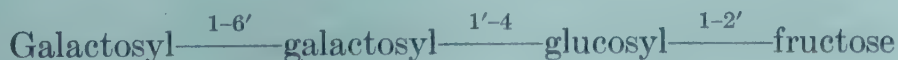
Other nonreducing oligosaccharides have been isolated from various plant sources. The trisaccharide melezitose, found in the sweet exudate of some trees, is made up of two glucose residues linked through a fructose as follows:



Sugar beets contain a small concentration of another trisaccharide, raffinose. In this compound galactose is condensed with a sucrose residue.



Stachyose is a tetrasaccharide which is widely distributed in plants and often occurs associated with raffinose. It is interesting therefore that the link between the galactose residue and that of glucose is different in the two sugars, being a 1-4' link in stachyose instead of the 1-6' link found in raffinose. Stachyose has the structure:



Verbascose is a pentasaccharide which occurs in some species of mullein and probably has the structure



REDUCING OLIGOSACCHARIDES

Most of the common oligosaccharides reduce Fehling's solution, show mutarotation in solution, and react with carbonyl reagents to form such

derivatives as oximes and osazones. In these compounds the sugar residues are united in such a way as to leave one potential carbonyl group free.

Lactose ($C_{12}H_{22}O_{11}$). Lactose or milk sugar is found in mammalian milk in a concentration of approximately 5 per cent. This is one of the few carbohydrates associated entirely with the animal kingdom, most carbohydrates being plant products. The formulation of its structure rests upon the following facts:

1. It is a reducing sugar and after hydrolysis of a given weight of lactose the reducing power of the solution is doubled. This indicates that a second carbonyl group has been set free and that the compound is therefore a glycoside.

2. Acid hydrolysis of lactose sets free equimolecular amounts of glucose and galactose.

3. Oxidation of lactose with bromine water yields lactobionic acid, which can be hydrolyzed to form gluconic acid and galactose. This indicates that in lactose it is the aldehyde group of glucose which is free to be oxidized, and the sugar is therefore a galactoside.

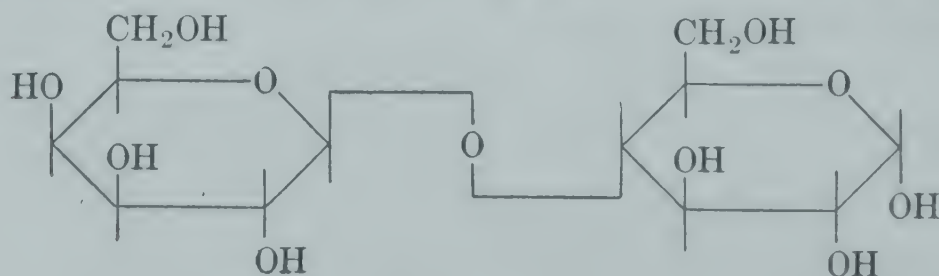
4. Complete methylation yields an octamethyl derivative which on hydrolysis liberates 2,3,6-trimethylglucose and 2,3,4,6-tetramethylgalactose. The eighth methyl group which was originally introduced into the sugar had been attached at carbon 1 of the glucose, and since it was thus held in a glucosidic link it had been set free when the compound was hydrolyzed. The positions of the methyl groups indicate that the galactose is present in pyranose form, and that lactose must have either one of the two following structures:

(a) Galactopyranose—^{1-4'}—glucopyranose

(b) Galactopyranose—^{1-5'}—glucofuranose

5. If lactobionic acid is methylated before it is hydrolyzed, one of the hydrolytic products is the lactone of 2,3,5,6-tetramethylgluconic acid. This means that in the lactobionic acid, in which there is no glucose ring, the only hydroxyl which was not free to be methylated was the one at position 4. This must therefore be the point of attachment of galactose to glucose and the disaccharide must have the structure (a) above.

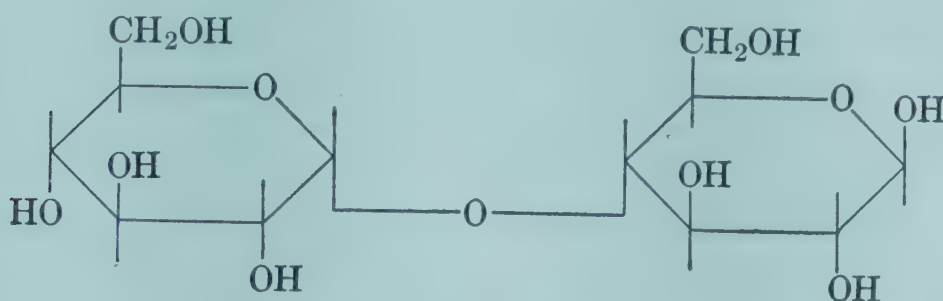
6. The fact that lactose is hydrolyzed by an enzyme which attacks β -galactosidic linkages indicates that the galactose has the β -configuration



Lactose

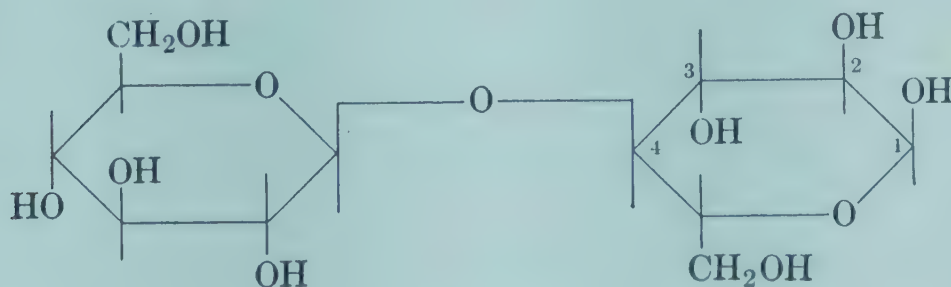
at the anomeric carbon. This is further confirmed by the low specific rotation of the sugar, ($[\alpha]_D +35^\circ \rightarrow +55^\circ$). Lactose is then 4- $[\beta$ -D-galactopyranosyl]-D-glucose or 4-D-glucose- β -D-galactopyranoside.

Maltose. The reducing disaccharide which forms in the course of hydrolysis of starch is maltose, which yields on further hydrolysis two molecules of glucose. Proof of its structure parallels that given above for lactose. Its reducing power is doubled on hydrolysis. Methylation studies show that in the two monose units carbon 1 of one glucose is linked to carbon 4 of the other, and that both hexose units have the pyranose ring. Its high specific rotation ($[\alpha]_D +112^\circ \rightarrow +130^\circ$) and its hydrolysis by maltase indicate the presence of an α -glucosidic link. It is then 4-D-glucose- α -D-glucopyranoside, as shown in the formula.



Maltose

Cellobiose. Of great importance in the elucidation of the structure of cellulose is the disaccharide cellobiose which can be isolated as an octaacetate if cellulose is simultaneously acetylated and hydrolyzed. The disaccharide itself can be obtained by deacetylation of this compound and proves to consist as does maltose of two glucose residues. It has however the low specific rotation ($[\alpha]_D +14^\circ \rightarrow +35^\circ$) characteristic of β -glucosides and is hydrolyzed by emulsin and not by maltase. In other respects it reacts exactly as maltose does, yielding the same products on methylation and hydrolysis. Its formulation as a β -glucoside has been fully confirmed by synthesis.



Cellobiose

Although these three important disaccharides are all constituted with a 1-4' linkage, it must not be assumed that this is the only type of bond by which monose residues are linked. In various natural glycosides there

occurs another disaccharide, gentiobiose, in which carbon 1 of one glucose is linked to carbon 6 of another. Melibiose, which results from partial hydrolysis of the trisaccharide raffinose, is made up of glucose united in a 6-1' linkage to galactose. Other oligosaccharides obtained by partial hydrolysis of more complex substances exhibit 1-2' and 1-3' links. In every compound so far studied, the hexose rings are pyranose except in fructose residues. This sugar assumes the furanose form not only in sucrose, but in several trisaccharides.

The Polysaccharides

As was indicated in Table 4-I, the polysaccharides serve as reserve food substances for both plants and animals, and as structural material in plants. They are defined as polymerized products in which monoses or their derivatives are united, usually by glycosidic linkages. The typical members of the group have very high molecular weights and as they are hydrolyzed to simpler and simpler molecules the products merge gradually into the group of oligosaccharides. The most important polysaccharides are cellulose and starch among plant products, and glycogen which is the reserve carbohydrate of animals.

Complete hydrolysis of the polysaccharides yields either monosaccharides or closely related compounds. The most common constituent is D-glucose, but several different pentoses and uronic acids as well as amino sugars and other hexoses occur in polysaccharide molecules. Some of these compounds yield on hydrolysis only one type of monosaccharide. They may then be named from the constituent sugar by replacing "ose," or sometimes simply the terminal "e," by "an." Thus starch, cellulose and glycogen, all of which are made up of glucose residues, are glucosans or *dextrans*. Polysaccharides which yield mannose on hydrolysis are *mannans* while those made up of fructose residues are *levans* (from levulose).

More complex than these compounds, and not as yet completely formulated, are a number of polysaccharides which are made up of more than one type of monose or monose derivative. Such for example are the gums and mucilages and certain specific polysaccharides of bacterial origin, all of which contain uronic acids linked to various simple sugars.

PROCEDURES FOR THE DETERMINATION OF POLYSACCHARIDE STRUCTURE

Because of their commercial importance starch and cellulose have been exhaustively investigated and the main outlines of their structures are now established. This has been achieved partly by the use of the procedures which elucidated the structure of glucose and partly by the use of special procedures. The presence or absence of free reducing groups is determined by familiar procedures, such as titration with iodine in sodium hydroxide or preparation of phenylhydrazine derivatives or estimation of

free carboxyl groups following gentle oxidation. Methylation establishes the number of unsubstituted hydroxyl groups while periodic acid titration will show whether or not these groups are adjacent to each other. For example, 1-6' glycosidic linkages may thus be differentiated from 1-4' links, since in compounds of the first type there are three adjacent unsubstituted hydroxyl groups on each hexose residue while in the second there are but two. The presence of free primary hydroxyl groups can be demonstrated either by the ready formation of a monotrityl derivative or by formation of a tosyl derivative in which the tosyloxy group can be replaced by iodine.

Complete acid hydrolysis of a polysaccharide determines the type or types of monosaccharide units of which it is built. Further light on the structure is often obtained by partial hydrolysis, usually catalyzed by enzymes. Isolation of a disaccharide from such a reaction mixture makes it possible to determine the nature of some of the glycosidic links. For example, maltose is freed when starch hydrolyzes in the presence of malt diastase. The presence of the α -1-4' link in this sugar indicates that in starch some of the glucose residues are united by this type of bond. Furthermore, in certain compounds some of the linkages prove more resistant than others to hydrolysis. In such cases determination of the type of sugar which is freed in the early stages of hydrolysis demonstrates which are in terminal positions and therefore exposed to early attack by enzymes.

The method of "end-group" analysis is a chemical procedure which has contributed greatly to the elucidation of polysaccharide structure. A number of monosaccharide units may be linked together in such a way that they form a long chain, no single residue being attached to more than two others, or the large molecule may be branched, some of the inner residues being attached to three others at what is known as a branching point. In the first type of polysaccharide the two terminal residues would differ from all the central ones in having at one end of the chain an extra free hydroxyl group and at the other a free reducing group. But if a polysaccharide has a branched structure the number of end residues is correspondingly increased and the number of free hydroxyl groups on internal residues reduced. Thus when a polysaccharide is first methylated and then hydrolyzed, internal monose residues between two others give rise to trimethyl derivatives. But those which have three links at a branching point will form only dimethyl substitution products, while terminal nonreducing units will give rise to tetramethyl derivatives.

The reducing terminal residues cannot be differentiated from internal ones with two links, since their fourth methyl substituent is glycosidic, and is lost during hydrolysis. Determination of the percentage of tetramethyl compound in the hydrolytic mixture indicates the number of nonreducing terminal units while the amount of dimethyl derivative is a measure of the number of branching points. By comparison with known

methyalted derivatives the positions of the methyl groups can be determined, and these serve to define the positions at which there were linkages in the original polysaccharide.

X-ray analysis is a physical method which has been widely used in structural studies, especially with cellulose. A fine beam of x-rays emerging from a slit in a lead screen passes through the material under examination. In the course of that passage it is diffracted by the atomic particles which it encounters, and the rays which emerge are then allowed to fall upon a sensitive plate. If the particles within the molecule are arranged regularly enough for one diffracted beam to reinforce another, the plate will show a diagram made up either of concentric circles or of symmetrical arcs or dots. Even though the polysaccharides do not have the regular arrangements characteristic of crystals, they do yield x-ray diffraction diagrams from which it is possible to calculate certain intramolecular distances, and also the dimensions of any units which are repeated regularly within the molecule. For example, from x-ray evidence it appears that the "unit cell" or repeating unit in the cellulose molecule contains four glucose residues and has the dimensions indicated in Figure 4.1 on page 140.

MOLECULAR WEIGHT DETERMINATIONS

Determination of the molecular weights of the polysaccharides has proved to be extremely difficult and there are still many unanswered questions. These substances as obtained from natural sources are far from homogeneous and the preliminary purification has undoubtedly been inadequate in many experiments. There is no clear criterion of purity, such as the melting point of crystalline compounds, and it has been necessary to decide on some indirect basis whether or not a given sample is homogeneous. In one study, for example, when it was found that corresponding molecular weights were given by purified cellulose, by several of its acetylated derivatives, and by the carbohydrates regenerated from these derivatives, it was assumed that the original sample had been uniform in composition.

The Ultracentrifuge. The instrument known as the ultracentrifuge was developed by Theodor Svedberg (1884—) at the University of Upsala. It is generally used at speeds which develop a field having more than 400,000 times the force of gravity. When a polysaccharide solution is centrifuged under these conditions in a suitable cell, the molecules settle out at a rate which depends in part upon their own weight. Various physical methods have been developed for measuring the concentration of particles at different levels in the cell, and from the distance traveled in a given time it is possible to calculate their weights. If a substance is homogeneous there comes to be a sharp moving boundary between the solvent and the upper layer of the dispersed material, and it is possible to follow

the rate at which this boundary moves. If on the other hand the sample is heterogeneous, the molecules of different weights will be spaced along the cell length, and the number of different layers is then an indication of the number of different molecular weights represented.

Other Methods. In spite of the fact that classical osmotic pressure determinations are difficult to apply to substances of high molecular weight, many polysaccharide molecular weights have been determined by this method.

The estimation of molecular weights by viscosity measurement depends upon the fact that the viscosity of substances of colloidal dimensions increases with the molecular weight of the solute. As originally elaborated this method depended upon empirical equations to calculate the molecular weight from the observed viscosity, but the method has since been put upon a firmer theoretical basis.

The small group of results brought together in Table 4-III show how heterogeneous some samples of starch and cellulose and glycogen must be,

TABLE 4-III. MOLECULAR WEIGHTS OF POLYSACCHARIDES

Substance	Method	Degree of Polymerization	Molecular Weight
Cellulose (untreated cotton)	Viscosity	2,020	330,000
Cellulose (bacterial)	Viscosity	1,890	310,000
Cellulose (purified)	Ultracentrifuge	1,000-3,000	150,000-500,000
Cellulose (raw)	Ultracentrifuge	6,200	1,000,000
Starch (potato amylopectin)	Osmotic Pressure	1,275	207,000
Starch (potato amylose)	Osmotic Pressure	500-800	82,000-130,000
Glycogen (rabbit liver)	Osmotic Pressure	12,000	2,000,000
Glycogen (purified)	Osmotic Pressure	9,000	1,500,000

and how far we are from being able to assign definite molecular weights to such substances. Indeed one definition of the polysaccharides describes them as "collections of polymeric homologues, each based on the same chemical ground plan, but displaying a range of molecular weights."

CELLULOSE

The heavy cell walls of plants are composed largely of cellulose which constitutes more than 50 per cent of woody tissue and more than 90 per cent of cotton. It is a glucose polymer for which the vertebrates have no

digestive enzymes, though some invertebrates are able to digest it. It is however a valuable foodstuff for herbivorous animals, thanks to the cellulose-splitting enzymes elaborated by the microorganisms of their digestive tracts.

Most of the structural studies of cellulose have been made on material derived from cotton, since it occurs there in a relatively pure form, and the further purification requires only gentle manipulation. The evidence

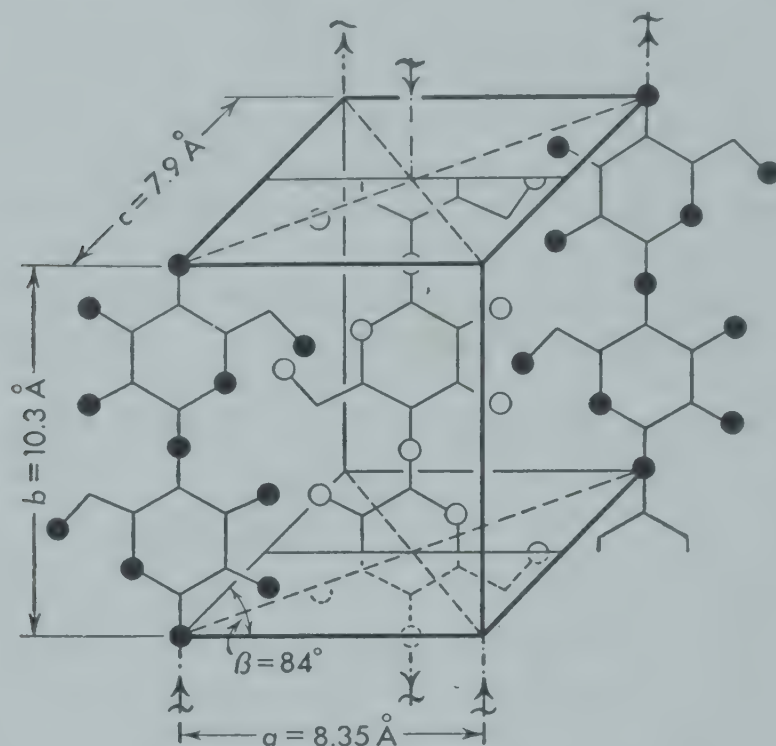


Figure 4.1. Diagrammatic representation of the unit cell of cellulose. Note that each unit cell contains four glucose residues. These include the two in the center of the figure and one-fourth of each of the eight which are placed at the corners of the cell. The corner residues are shared by each of the four unit cells which meet at these corners. Of the eight residues which make up the corners of the cell in the diagram only four are represented. Similar pairs at the other two corners complete the cell. (From K. H. Meyer and L. Misch, *Helv. Chim. Acta*, 20:232, 1937.)

points to a structure in which more than 2000 β -glucose residues are linked to form a long chain molecule. This formulation is based upon the following evidence.

1. The empirical formula is $(C_6H_{10}O_5)_x$.
2. Complete hydrolysis yields only D-glucose.
3. Formation of triacetates and trimethyl ethers indicates that each glucose residue has three unsubstituted hydroxyl groups. The fact that the yield of these compounds is practically quantitative means that the molecule has almost no end-groups and is therefore a long straight chain of glucose residues.
4. Formation of a monotosyl derivative which reacts with sodium iodide to replace the tosyloxy group with iodine, shows that each residue has one unsubstituted primary alcohol group.

5. When cellulose is acetylated and hydrolyzed the only product other than acetylated glucose is the octaacetate of the disaccharide cellobiose. This compound has been shown to be a β -1-4'-glucoside, hence it is concluded that the cellulose chain is linked in this same way.
6. As previously noted, the molecular weights obtained with cellulose vary widely, but it is clear that the chain must consist of 1000 to 2000 glucose units.

Figure 4.1 gives a spacial diagram of the repeating unit in cellulose. This is believed to represent part of a crystalline area in a cellulose fiber, such areas being intermingled with amorphous regions in which the chains are less symmetrically disposed. The exact orientation was determined by comparing actual x-ray diagrams formed by purified cellulose with those to be expected from various spacial models. It should be noted that adjacent chains extend in opposite directions in this formulation, with the oxygen of the ring at the "upper" part of the hexagon in one chain and at the "lower" end in the neighboring chain. With chains thus disposed in close proximity it is assumed that cross linkages occur in the form of hydrogen bridges, thus giving to cellulose its great stability.

STARCH

A more complex polysaccharide than cellulose is another dextran, starch, which occurs in the form of characteristic granules in seeds, roots, and fruits. It serves as a reserve food either for the plant itself, which stores in this form any excess carbohydrate formed in photosynthesis, or for the new plants which will form when the starch-packed seeds germinate. Indirectly of course it also constitutes a generally useful food supply for animals, since it differs from cellulose in being easily split by digestive enzymes.

Starch as extracted from different plants is not a single individual, but differs slightly from plant to plant, being in all cases a complex mixture of related substances. On the basis of end-group assay and of methylation studies it was originally formulated by Haworth as a short chain molecule containing 25 glucose residues held in 1-4'-glucosidic bonds. This structure was based on the formation from starch of methylated glucose derivatives of which approximately 90 per cent was 2,3,6-trimethylglucose and 4.5 per cent was 2,3,4,6-tetramethylglucose. Since partial enzymic hydrolysis of starch yields largely maltose, the glucose residues are believed to be linked as in maltose by α -glucosidic bonds. This view is supported by the high optical rotation of starch derivatives.

From the first there were objections to this simple formulation. In the first place, the molecular weight of starch was known to be far larger than the 4000 which would represent about 25 glucose residues. More-

over, in hydrolysis of the polysaccharide by the starch-splitting enzyme, β -amylase, only about 70–80 per cent of the starch was transformed to maltose. The unexplained residue, known as “limit dextrins,” was of much lower molecular weight than the original starch.

Meantime it began to appear that “starch” is in fact a mixture of at least two components, now known as amylose and amylopectin. Many methods of separating these substances have been devised, but one of the simplest depends upon precipitation of the amylose fraction. The starch is first dispersed in water by autoclaving. Saturation of the resulting solution with butanol or cyclohexanol causes a very nearly quantitative separation of the amylose, leaving the amylopectin in solution. In Table 4-IV are brought together the properties of the two substances, on which depend their identification as separate entities.

TABLE 4-IV. PROPERTIES OF THE TWO MAIN CONSTITUENTS OF STARCH

	Amylose	Amylopectin
Color with iodine	Deep blue	Red to purple
Stability of solutions	Yields gels which “retrograde”	Solutions stable
Molecular weights	Usually not greater than 50,000	Greater than 200,000
Approximate number of glucose units per mole- cule	300	1300
Proportion of end-groups	1:300	1:27
Effect of β -amylase	Complete hydrolysis to maltose	Hydrolysis to extent of about 50 per cent, leaving a nonreducing limit dextrin of mol. wt. of about 80,000

For amylose, which in most starches makes up about 20 per cent of the total, end-group assay indicates that only one glucose unit in every 300 is capable of forming tetramethylglucose. Since molecular weight estimations show that approximately 300 glucose units make up the amylose molecule, it is evident that it must consist of a single straight chain of glucose units. The hydrolysis of amylose by β -amylase results in its complete transformation to maltose, hence the linkages are believed to be α -glucosidic. This picture of amylose as a long slender chain explains many known properties of the substance. On cooling a hot solution of amylose it sets to a gel very rapidly. This may well result from formation of a network of interlacing, randomly distributed linear molecules. But when such a solution is allowed to stand, the solute slowly deposits as a fine crystalline mass. This process is known as *retrogradation* of the starch, and might be expected if similar slender molecules gradually line

up in such a way that they can form loose aggregations which are too large to remain in solution.

It has been suggested that the glucose chains in amylose differ from those in cellulose in having the ability to coil in the form of a helix (Fig. 4.2) in which there are six glucopyranose units in each turn. X-ray studies support this structure and it is of interest to note that while the α -glucosidic form of amylose lends itself to such a coiling, a straight chain of glucose units is enforced upon cellulose by the β -configuration at the anomeric carbons in that compound.

The helical formulation of amylose has been especially useful in explaining the intense blue color given by amylose with iodine. The inner dimensions of the spiral which has been postulated are such that an iodine molecule can just be fitted inside. It is therefore suggested that the starch-

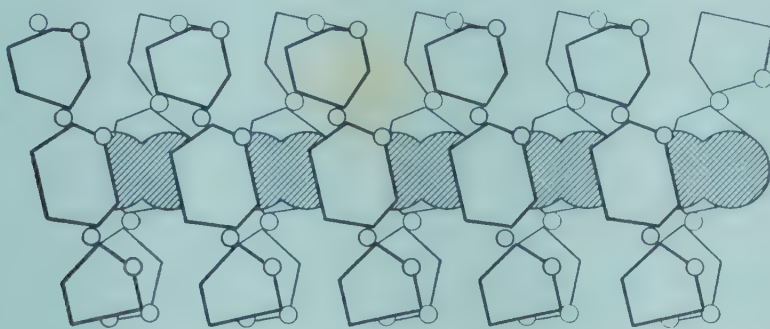


Figure 4.2. Diagram of a helical starch chain with iodine molecules in the center of the helix. (From R. E. Rundle and R. R. Baldwin, *J.A.C.S.*, 65:555, 1943.)

iodine complex may consist of iodine molecules lining the center of the helix, with one iodine molecule associated with each turn.

Molecular weight estimations and end-group assay of amylopectin point to a molecule made up of over 1000 glucose units, so arranged that there is a nonreducing end-group for every twenty to twenty-six residues. Various types of branched or laminated molecules have been postulated, but the multiple-branched form suggested by the late Kurt Meyer of the University of Geneva is now widely accepted (Fig. 4.3). Support for this structure is found in the behavior of amylopectin with β -amylase, which hydrolyzes only about half of the molecule to maltose. The residue consists of limit dextrins, polysaccharides having molecular weights of approximately 80,000 and very highly branched chains, i.e., one end-group for every eleven or twelve glucose residues. It is believed that β -amylase attacks the free ends of the glucose chains, splitting off maltose units until it meets a variation in the pattern such as would be offered by a 1-6' link at a branching point of the chain. The dextrins would then consist of the truncated, highly ramified molecules left when the enzyme has come as close as it can to each branching point. As indicated in the figure, Meyer suggests that a second enzyme, α -glucosidase, is able to act upon the dex-

trin because it is able to break the link at the branching point, thus freeing the chains of the remaining dextrin so that they can be attacked again by β -amylase. Assuming this branched structure for amylopectin, its stability in solution depends on such molecules offering no opportunity for close aggregations. In solutions of whole starch the amylopectin probably acts as a protective colloid for the less stable amylose.

Although amylose and amylopectin constitute probably 99 per cent of the starch complex, the remaining 1 per cent varies from starch to starch and may be important in determining the structure of the starch grains. For example, the water which is present in the granules seems to play a



Figure 4.3. Diagrammatic representation of amylopectin. Each small circle stands for a glucose residue. The outermost dotted line represents the point to which β -amylase degrades such a molecule. The short lines mark the extent of the hydrolysis by α -glucosidase which has split the 1-6' bonds. A second treatment with β -amylase degrades the chains until new branching points are reached as indicated by the inner dotted line. A = the reducing end of the molecule. (From K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, 23:880, 1940.)

part in their crystalline structure, for when it is removed the crystalline pattern disappears. Most starches are also associated with small amounts of fatty acid, but whether they are really combined chemically or merely adsorbed is not clear. The same question arises in relation to the phosphorus which occurs in native starch grains. From some starches it can be washed away in the form of soluble phosphate compounds by repeated extraction with warm water. In others, of which potato starch is an example, the phosphate seems actually to be combined with the starch itself. In general the root starches include phosphorus esterified with the starch itself, while the phosphate of cereal or seed starches is in combination with smaller, soluble molecules. The functions of these traces of noncarbohydrate substances in starch grains is unknown.

GLYCOGEN ($C_6H_{10}O_5$)_x

Animal carbohydrate reserves are stored largely in liver and muscles in the form of the polysaccharide glycogen. Although hydrolysis and end-group assay indicate a molecule containing twelve to eighteen glucopyranose units in a chain, the molecular weight of glycogen is certainly more than 1,000,000. As with amylopectin this discrepancy is explained

if glycogen is formulated as a multiple-branched molecule (Fig. 4.4). Hydrolysis with β -amylase transforms about 50 per cent of the polysaccharide to maltose, leaving a product of high molecular weight in which the average chain length has dropped to 5.5 glucose units. Meyer and Fuld interpret this to mean that each branch in glycogen consists of six to seven glucose units joined by 1-4' glucosidic links, and that β -amylase shortens each such chain to one or two units by removing successive maltose molecules from the free ends. At this point the enzyme is halted by the new configuration at the branching point where it reaches a 1-6' link, and there is left a truncated polysaccharide residue. In the figure the short distances between branching points indicate that there are believed to be only about three glucose units between branches.

MISCELLANEOUS POLYSACCHARIDES

Homopolysaccharides. Although the three most important polysaccharides are all glucose polymers with 1-4' and 1-6' linkages, this uniformity does not mean that all polysaccharides are built on the same pattern. From lichens, yeast, and bacteria there have been isolated other dextrans which differ from cellulose and starch either in having different types of linkages or very different branch lengths. Fructose polymers or levans are widely distributed among plants. One of these is inulin, the polysaccharide of the dahlia tuber, which has found a use in medicine in kidney function tests, since it is nontoxic and can be safely injected into the circulation. It is completely filtered into the glomerular filtrate in the kidney and is not later reabsorbed in the lower tubule, hence the rate at which the injected material is transferred to the urine is a measure of the rate of glomerular filtration. The pectic substances comprise a group of closely related compounds which are essential constituents of plant cell walls. They include a *protopectin*, insoluble in water and found only in cell walls, *pectin* which is a neutral water-soluble substance found in plant extracts, and *pectic acid* which is formed when pectin is hydrolyzed with dilute alkali. Pectic acid is believed to consist of approximately one hundred galacturonic acid residues united in α -glycosidic linkages through the 1-4' positions. In such a structure the carboxyl groups are free, and addition of calcium, barium, or strontium ion precipitates a rigid gel in which the divalent cation probably links together two acid chains.

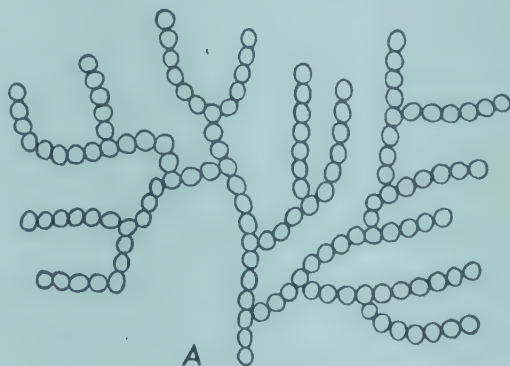
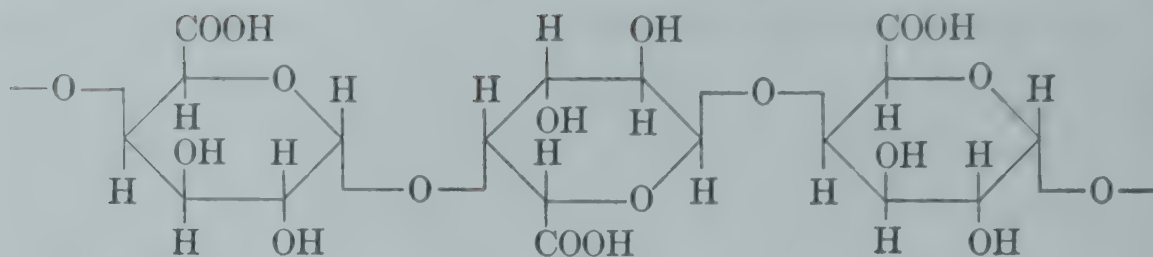


Figure 4.4. Diagram of the structure of glycogen. The one reducing group represented is at A. Each circle indicates a glucose residue. (From K. H. Meyer and M. Fuld, *Helv. Chim. Acta*, 24:376, 1941.)



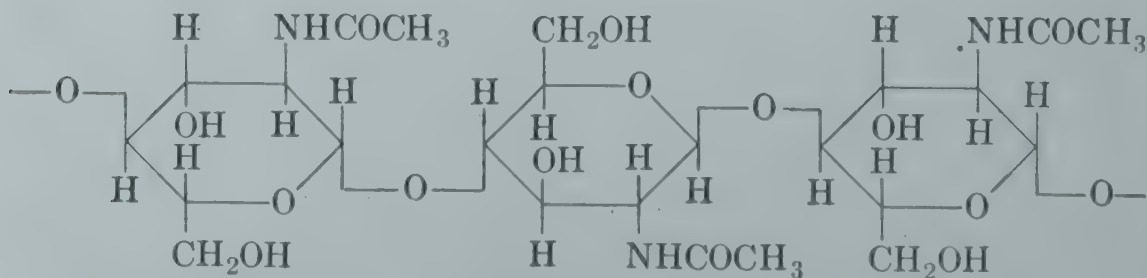
Portion of a pectic acid chain

The term "pectin" (or pectins) is applied to the water-soluble methyl esters of pectic acid which result from partial hydrolysis of protopectin with acid. In the pectin of commerce most of the carboxyl groups are esterified, hence the compound is neutral and cannot be precipitated with calcium or other divalent cation. The commercial use of pectin depends upon its ability to form a rigid gel with sugar and acid.

Protopectin as its name indicates is considered the parent substance of this group, yielding pectin or pectic acid on partial hydrolysis. There is some evidence that protopectin consists of methyl polygalacturonide chains much longer than those of pectin, containing perhaps a thousand residues per chain. On the other hand recent work suggests that the compound may be a giant molecule made up of pectin chains linked together through divalent metal ions or through esterification of two chains with a single phosphoric acid molecule to give a three-dimensional network.

The pectic compounds occur associated with galactans (galactose polymers) and arabans (arabinose polymers) but whether these polysaccharides are chemically combined with the polygalacturonide is not yet clear. Evidence is accumulating that the pectin molecule has side chains and these may well be arabans and galactans held in ester linkages.

Reference has already been made (p. 123) to chitin, the polysaccharide which is the organic skeletal substance of insects, crustacea, and fungi. This is a polymer of N-acetylglucosamine, probably having 1-4' β -glycosidic linkages. The compound thus differs from cellulose only in having an acetylated amino group instead of an hydroxyl group on carbon 2 of the glucose ring.



Portion of a chitin chain

Heteropolysaccharides. In both the plant and animal world there are found many polysaccharides which on hydrolysis yield more than one

monosaccharide type. Associated with cellulose and lignin in the cell walls of plants are a number of compounds known collectively as the hemicelluloses. Some of these are simple pentosans or hexosans which form part of the cellulose micelle. Others are amorphous in nature and are found in association with lignin deposited in the interstices of the cell wall. These latter are known as the *polyuronide hemicelluloses*, though they yield on hydrolysis not only uronic acid but one or more simple sugars. They thus belong to the group of heteropolysaccharides. They consist of mixed glycosidic chains in which pentose and uronic acid molecules are combined. For example, the hemicellulose of wheat straw contains uronic acid, arabinose, and xylose in the approximate ratio of 1:1:23. The solubility of the individual polysaccharide depends upon this ratio, the less soluble ones containing one uronic acid to eighteen to twenty pentose units, while in the more soluble fractions there is one uronic acid to eight to twelve pentose residues. Many of the hemicelluloses contain methoxyl groups, probably in esterified carboxyl groups of the uronic acids.

Plant *gums* which occur in exudates, probably pathological, on the bark and fruit of trees, make up another group of polysaccharides in which uronic acids occur in combination with simple sugars.

The mucopolysaccharides constitute a heterogeneous group of compounds which occur in loose combination with protein. Various animal fluids such as synovial fluid or vitreous humor contain the mucopolysaccharide, *hyaluronic acid*, which yields on hydrolysis equimolecular proportions of glucosamine, glucuronic acid, and acetic acid. A similar polysaccharide is *chondroitin sulfuric acid* which may be isolated from cartilage. Hydrolysis shows it to be a mixed polymer of glucuronic acid and 2-desoxy-2-aminogalactose (chondrosamine) in which the amino group is acetylated, and sulfuric acid is esterified at some point. Liver extract yields a third member of this group, the anticoagulant *heparin*. Like chondroitin sulfuric acid it is a sulfate ester of a polysaccharide, but includes acetylated glucosamine in place of chondrosamine. Its anticoagulant powers are believed to reside largely in the sulfate groups, for sulfate esters of other polysaccharides exhibit similar properties. It should not be concluded at this point that heparin and chondroitin sulfuric acid differ only in the configuration of the amino sugar, since neither the structure nor the molecular weight of either substance is now known.

Perhaps the most interesting of the heteropolysaccharides are those which are produced by microorganisms and which have immunological properties. The immunity which is conferred upon a person who has had such a disease as smallpox resides in the presence in his blood stream of protective substances called *antibodies*. These form in response to the presence in the patient of a foreign substance elaborated by the infective agent and known as an *antigen*. The relation between the antibody and

the antigen can be demonstrated experimentally. If to one sample of serum from the immune person there is added an extract of the infective agent a precipitate will form. But if the immune serum is tested with another type of organism there will be no flocculation. This indicates that an antibody is specifically tailored to fit in some way the particular invader with which it has to cope, and that its own "shape" does not allow it to react in the same way even with molecules which closely resemble its antigen in structure.

It has been found that the polysaccharides elaborated by pneumococci and present in the blood and urine of pneumonia patients are responsible for the immunological specificity of the bacteria. Since then other bacteria, including *B. dysenteriae* and tubercle bacilli have been shown to produce immunologically specific polysaccharides. Some of these are themselves antigenic and stimulate formation of antibodies if injected into animals. Others show that they contain the specific groups involved in the precipitation, for they do cause flocculation of the corresponding antisera, but are unable to stimulate antibody formation. They become true antigens only in combination with protein. Many of these immuno-polysaccharides contain uronic acids and amino sugars, but their constituents vary widely, some containing no nitrogen while others contain bound phosphorus. A complete elucidation of their structure will be of great interest both to chemists and to bacteriologists.

Suggestions for Further Reading

GENERAL

GILMAN, H. (ed.), *Organic Chemistry: An Advanced Treatise*, 2nd ed., Wiley, New York, 1943.

These volumes contain three excellent chapters devoted to carbohydrate chemistry.

HAWORTH, W. N., *The Constitution of the Sugars*, Arnold, London, 1929.

In this delightful small book is summarized, by one who took a major part in the work, those experiments which established the structure of the simple sugars.

HUDSON, C. S., *Collected Papers*, Hann, R. M., and Richtmeyer, N. K. (eds.), Academic, New York, 1946.

McILROY, R. J., *The Plant Glycosides*, Arnold, London, 1951.

PERCIVAL, E. G. V., *Structural Carbohydrate Chemistry*, Muller, London, 1950.

This book does for 1950 what Haworth's book did 20 years earlier. It is called a textbook, but the author's style and his historical perspective give it a charm which is rarely found in texts.

PIGMAN, W. W., and GOEPP, R. M., JR., *Chemistry of the Carbohydrates*, Academic, New York, 1948.

This is a very complete compendium which fully justifies its broadly inclusive title.

PIGMAN, W. W., and WOLFRAM, M. L. (eds.), *Advances in Carbohydrate Chemistry*, Academic, New York.

This series began in 1945. The yearly volume carries authoritative articles on various phases of carbohydrate chemistry. The most recent volumes are very specialized, but the earlier ones have sections of general interest, to some of which references are given below.

PAPERS OF HISTORICAL INTEREST

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"Methods for the Determination of Reducing Sugars in Blood, Urine, Milk and Other Solutions," *J.B.C.*, 45:365, 1921.

SOMOGYI, M., "Notes on Sugar Determination," *J.B.C.*, 70:599, 1926.

This paper adapts the Schaffer-Hartmann method to the estimation of smaller amounts of glucose than could be determined with the original solutions.

"Notes on Sugar Determination," *J.B.C.*, 195:19, 1952.

This paper describes improved copper reagents.

Study Questions

1. Write the formulas of the compounds formed when glucose reacts with: hydrocyanic acid, phenylhydrazine, hydroxyl amine, bromine water, concentrated nitric acid, a reducing agent, acetone.

2. What is the Kiliani reaction and what is its special importance?

3. Define: enantiomorphs; epimers; anomers.

4. What is the "D-series" of sugars? Why is levorotatory fructose a member of this series?

5. What is mutarotation and how is it explained?

6. Write formulas for an acetal and a hemiacetal of acetaldehyde; for α -methyl-D-fructoside; for β -methyl-L-glucopyranoside.

7. What substances form when glucose stands in a weakly basic solution, and by what mechanism are they believed to arise?

8. What products are formed when periodic acid reacts with sorbitol?

9. Write the formulas of the products formed when periodic acid reacts with α -methyl-D-glucopyranoside.

10. Write formulas for: the Cori ester, the Neuberg ester, and the Robison ester.

11. With what reagent does glucose react to form a "tosyl" ester, and what is the importance of these compounds? Give the formula for the reagent and indicate in abbreviated form the compound which it forms with glucose.

12. Why was the Purdie and Irvine reagent of such importance in the history of sugar chemistry? What reagent is now commonly used to prepare the same sort of derivatives?

13. Write the formula for 2-desoxy-D-ribose; for an amino sugar. Give one compound of biological importance in which each of the above occurs.

14. What is a "trityl" derivative? Give the formula of the reagent. What is its importance?
15. What is a glycoside? a glucoside? an aglycone?
16. If you had isolated a new polysaccharide how would you attack the problem of determining its structure? What could you learn by use of toluenesulfonyl chloride? by end-group analysis? by complete methylation?
17. What is the structure of glycogen? of the amylose fraction of starch? of amylopectin?
18. Write formulas for each of the following, using the ring type of formula wherever it is suitable: glucose-1-phosphate; glucuronic acid; gluconic acid δ -lactone; fructose diphosphate; ascorbic acid; inositol; maltose; sucrose.

The Compounds of Nitrogen

Since, in one way or another, the proteins are involved in all the chemical processes in living organisms, one may expect discoveries of fundamental importance to biological chemistry to result from the elucidation of their structure and reactions.

EMIL FISCHER (1906)

The Amino Acids and the Proteins

The proteins occur in all living cells and indeed constitute the major part of their solid contents. They were named by the Dutch biochemist Mulder who first pointed out that various samples of the colorless, amorphous, nitrogen-containing material which could be obtained from both plant and animal tissues exhibited certain basic similarities. He expressed his belief in the fundamental importance of this material by calling it "protein" from a Greek word meaning "of first importance."

PROTEIN CHARACTERISTICS

The protein group includes a wide variety of substances which appear superficially to have few properties in common. Horn and hair consist largely of protein, and so does egg white and milk curd. In spite of the obvious differences between such representative substances as these, they do have certain properties which justify their inclusion in a single chemical classification.

In the first place they have, as Mulder suspected, a unique place in the living economy. They form the structural basis of every living cell, and no organism from the smallest bacterium to the largest mammal can continue to live unless it is furnished material from which it can build cell proteins. All the enzymes elaborated by living cells are protein in whole or in part. The proteins differ from the lipids and the carbohydrates in that they are not stored to any appreciable extent, except in eggs or seeds where they are used in the formation of new tissue. Nor are they used primarily as sources of energy as are both fats and carbohydrates. Ingested protein is digested, part of it is built into the proteins of the organism and each day's excess of nitrogen is excreted. It is true, as we shall see, that the nonnitrogenous parts of the protein of the diet are ultimately used as sources of energy. But it is only on a high protein diet

that these fragments contribute significantly to the energy requirements of the organism, and if they are stored for future use they are stored, not as protein, but as fat or carbohydrate.

Even more striking is the specificity of the body proteins. Glucose is glucose whether it comes from a plant or an animal. The general structure of the simple fats is the same whatever their source, all being glyceryl esters of fatty acids. But each species of animal builds its own specific proteins, very like—but clearly distinguishable from—the proteins of every other species. No variations of diet influence in the slightest the structure of this specific pattern. The protein of duck egg is “foreign protein” to an animal of another species, even though it is chemically indistinguishable from the egg protein of that other species.

Not only do the proteins have these unique characteristics which set them in a class apart, but among themselves they exhibit certain rather striking similarities. In his 1839 paper Mulder noted that proteins obtained from different plant or animal sources had very similar elementary compositions. Since then this has been found to be true of all members of the class. Indeed, so constant is the percentage of nitrogen in a wide variety of different proteins that where great accuracy is not required, the amount of protein is calculated directly from the amount of nitrogen estimated. Table 5-I gives the ranges found in the elementary analysis

TABLE 5-I. AVERAGE ELEMENTARY COMPOSITION OF
SOME REPRESENTATIVE PROTEINS

Constituent	Percentage
Carbon	50–55
Hydrogen	6.8–7.3
Nitrogen	15.5–18.7
Oxygen	20–23
Sulfur	0.3–1.7
Phosphorus	about 0.8

of a great number of proteins. Remembering that such dissimilar substances as sponge, hair, muscle, and egg white are largely protein, the constancy of composition shown is certainly very striking.

A second way in which all proteins are alike is in being substances of very high molecular weight which, if they dissolve at all, form colloidal solutions. Being colloidal, their molecular weights cannot readily be determined in the ordinary way, but until very recently had to be derived indirectly. Hemoglobin, for example, contains about 0.33 per cent of iron. Each molecule of the protein must contain at least one atom of iron, which means that it must be of such a size that 56 atomic weight units are only 0.33 per cent of the whole. On this basis, by a simple proportion it is found that hemoglobin must have a molecular weight of at least 16,670.

$$x:56::100:0.33$$

$$x = \frac{5600}{0.33} = 16,670$$

Since this calculation was first made, many newer methods have come into use for determination of molecular weights, but it is still impossible to give reliable figures for proteins. Few proteins have been prepared in an unquestionably pure state. Some, once believed to be pure individuals, have recently been shown to consist of mixtures of very similar substances. The best figures available indicate that the molecular weights of some proteins may be as low as 10,000 or, with the viruses for example, as high as 15 or 20 million. One of the milk proteins, a lactoglobulin, which has been isolated as an apparently pure, crystalline compound, has a molecular weight of about 36,000.

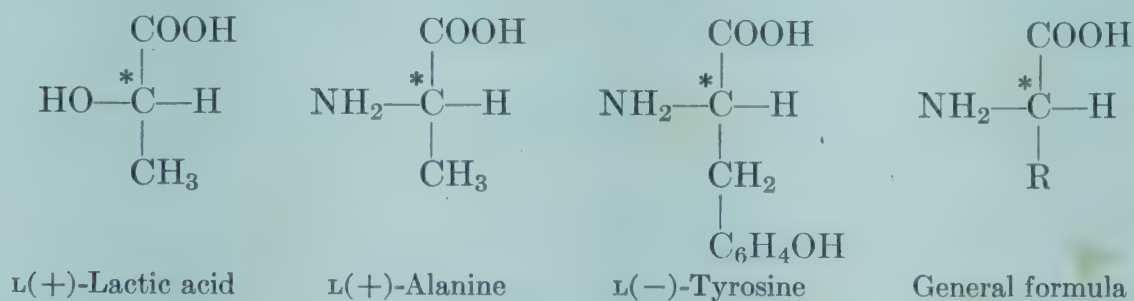
Certain proteins are readily dispersed in water or in dilute salt solutions, or in dilute acids or alkalis. These "soluble" proteins are all very similar in their response to various reagents and procedures. Many of them coagulate and precipitate as egg white does when their solutions are heated. Most soluble proteins can be precipitated by strong acids, or by solutions of heavy metal salts. The group of substances known as "alkaloidal reagents" which includes trichloroacetic acid, picric acid, tungstic acid, and others, brings about precipitation of most proteins. Many proteins can also be "salted out" of solution by greatly increasing the concentration of neutral salts such as ammonium sulfate or magnesium sulfate. All these procedures are general enough to justify grouping the reagents as "protein precipitants."

Finally, the proteins show their kinship most clearly in the fact that they all yield mixtures of α -amino acids on complete hydrolysis. Furthermore in view of the wide variety of proteins, the total number of different α -amino acids which have been proved to be protein constituents is remarkably small. Twenty-two or twenty-three are currently accepted as true products of protein hydrolysis. From these few building blocks living cells synthesize all their diverse proteins by varying the acids used, their ratios to each other, and their positions within the molecules. Before attempting to unravel the complexity of such molecules it will be necessary to gain an understanding of the properties of these fundamental simple molecules, the amino acids.

THE ALPHA AMINO ACIDS

Excepting only proline and hydroxyproline, all of the acids which are known to be constituents of the common proteins are α -amino acids. Except for glycine (α -amino acetic acid) all have centers of asymmetry at

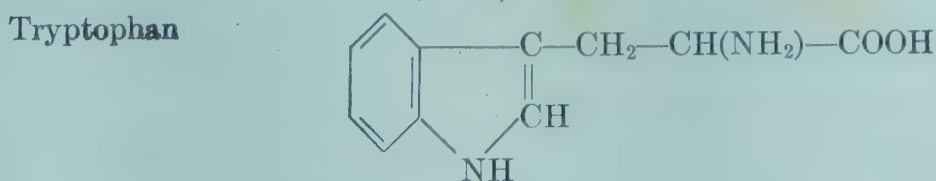
the alpha carbon, and occur in an optically active form. The actual sign of rotation varies from acid to acid, and indeed the rotation of a single acid may change with change in pH. But it has been shown that all have the same stereochemical configuration. That is to say, the spacial relation to each other of the amino group, the carboxyl group, the hydrogen, and the fourth or "R" group is identical in all the acids. Since this configuration is similar to that of L(+)-lactic acid, the natural amino acids are said to belong to the L-series.

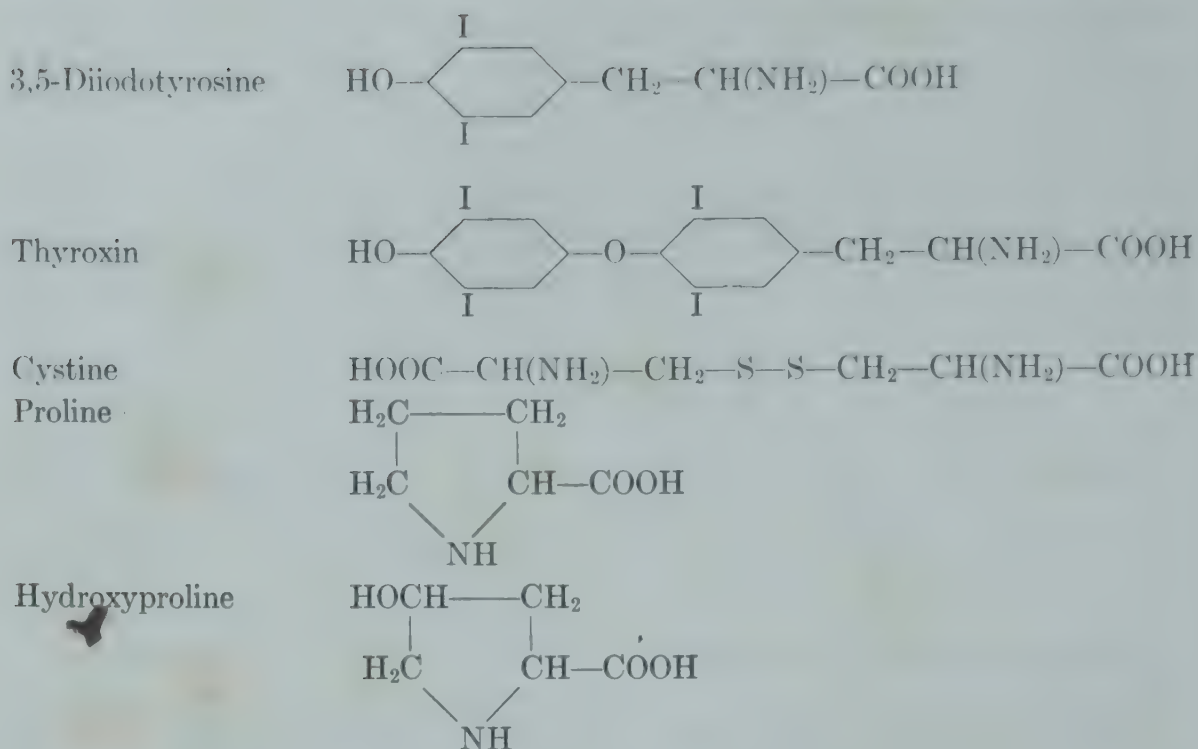


Classification of the Amino Acids. The natural amino acids fall into three groups, one of which comprises the neutral acids, the second the basic ones, and the third those which are acid in reaction. In the following classification are listed the α -amino acids which are believed to be constituents of native or unchanged proteins.

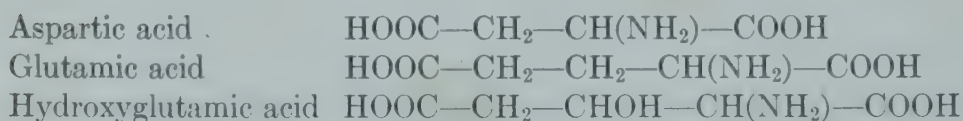
1. *The Neutral Acids; Monoamino Monocarboxylic Acids:* This is by far the largest of the three groups. Its neutral character depends upon the fact that the basicity of the amino group is roughly balanced by the acidity of the carboxyl group.

Glycine	$\text{CH}_2(\text{NH}_2)-\text{COOH}$
Alanine	$\text{CH}_3-\text{CH}(\text{NH}_2)-\text{COOH}$
Valine	$(\text{CH}_3)_2\text{CH}-\text{CH}(\text{NH}_2)-\text{COOH}$
Leucine	$(\text{CH}_3)_2\text{CH}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
Norleucine	$\text{CH}_3-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
Isoleucine	$\text{CH}_3-\text{CH}_2-\text{CH}(\text{CH}_3)-\text{CH}(\text{NH}_2)-\text{COOH}$
Phenylalanine	$\text{C}_6\text{H}_5-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$
Serine	$\text{CH}_2\text{OH}-\text{CH}(\text{NH}_2)-\text{COOH}$
Threonine	$\text{CH}_3-\text{CHOH}-\text{CH}(\text{NH}_2)-\text{COOH}$
Cysteine	$\text{CH}_2(\text{SH})-\text{CH}(\text{NH}_2)-\text{COOH}$
Methionine	$\text{CH}_2(\text{SCH}_3)-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$

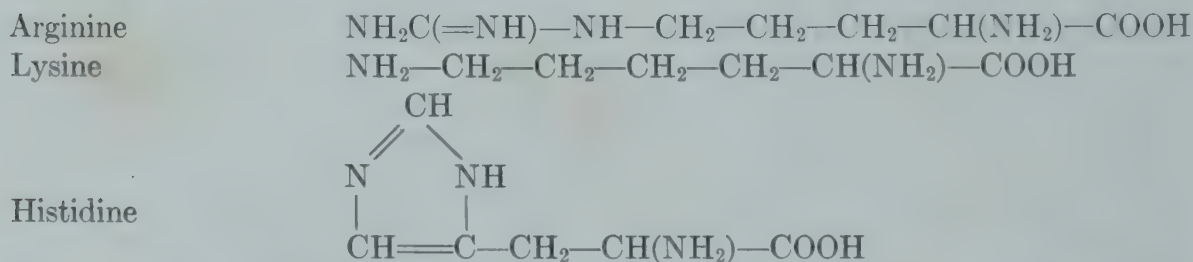




2. The Acidic Acids; Monoamino Dicarboxylic Acids:

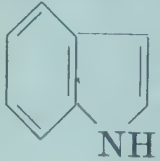


3. The Basic Acids:



For all the neutral amino acids except the two prolines the general formula $\text{R}-\text{CH}(\text{NH}_2)-\text{COOH}$ may be used. In glycine the R stands for hydrogen, and in six other neutral acids it stands for a simple alkyl or aryl group. The reactions of these seven acids will depend only upon the two functional groups, the amino group and the carboxyl group. The remaining members of this class possess other functional groups. Their reactions will accordingly include the reactions characteristic of those groups. Tyrosine has the properties of a phenolic hydroxyl group and cysteine those of a sulfhydryl ($-\text{SH}$) group. Proline and hydroxyproline are exceptional, both in structure and solubility. The α -amino group in these acids is involved in ring formation, and the acids have therefore anomalous properties.

The acidic amino acids have two carboxyl groups and but one amino group. This gives these compounds an acid reaction in water solution. The basic amino acids are those which contain more than one basic nitrogen grouping. In lysine this consists of a second free amino group; in arginine it is a guanido group. Since the substance guanidine ($\text{NH}_2\text{—C(=NH)—NH}_2$) is as strong a base as sodium hydroxide the basicity of this group is marked. The imidazole group in histidine is, on the other hand, only very slightly basic, so that histidine resembles the neutral acids in solubility. Note that there is a fourth acid which contains more than one nitrogen. This is tryptophan which contains an indole group but is listed with the neutral acids.

This is in accord with the fact that indole itself,  is not basic, but very slightly acidic in reaction.

Physical Properties of Amino Acids. The amino acids have physical properties which are very different from those of unsubstituted fatty acids. Saturated fatty acids above butyric are either only slightly soluble in water or are indeed quite insoluble. They are on the other hand very soluble in such an organic solvent as ether. They are either liquids at room temperature, or low-melting solids. Stearic acid, with eighteen carbons, melts at 69°C . In sharp contrast to these, the corresponding α -amino acids are extremely soluble in water and quite insoluble in ether. They are nonvolatile and they melt only with decomposition at temperatures above 200°C . These properties are reminiscent of those we associate with the crystal lattices of inorganic salts, held together by electrostatic attraction. It is now believed that the aliphatic amino acids exist largely in the form of molecules which may be thought of as salts. The neutralization of the acidity has taken place within the molecule, yielding an "inner salt," $\text{NH}_3^+\text{—CH(R)—COO}^-$. Such a self-neutralized molecule is known as a "dipolar ion" or a *zwitterion* from the German word meaning "hybrid." The molecule is believed to exist in this form in water solution also, thus explaining the fact that in an electric field the neutral amino acids do not move toward either electrode.

Chemical Properties of the Amino Acids. Chemically the amino acids have the properties of their two functional groups, though those of the carboxyl group in particular are somewhat modified by the proximity of the basic amino group.

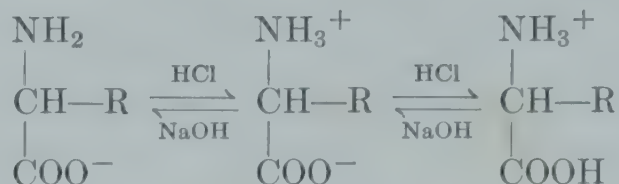
1. *Reactions with Acids and Bases:* When a mineral acid is added to the solution of an amino acid, the hydrogen ion gradually neutralizes the negative charge on the dipolar ion, until finally the acid exists entirely as a cation.



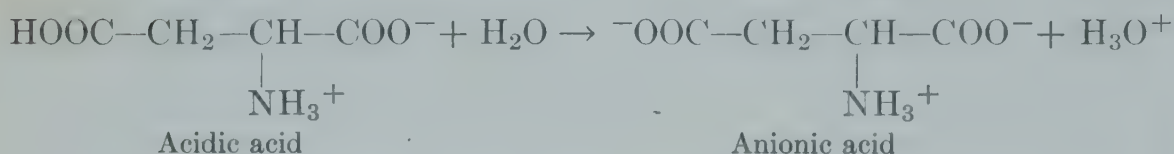
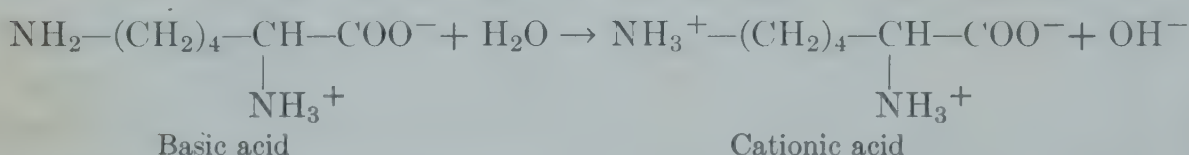
Similarly the addition of alkali brings about the loss of the positive charge and the formation of an anion.



Thus the three forms, anion, cation, and dipolar ion, are freely interconvertible.

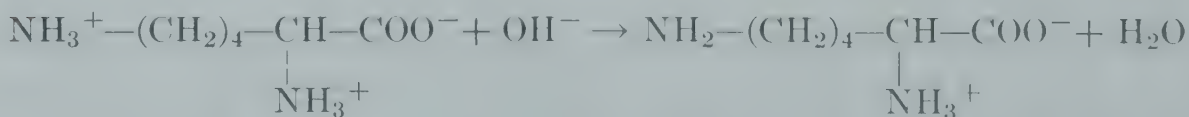


2. *Behavior in an Electric Field:* It has been noted that if a solution of neutral amino acids is subjected to an electric field, their dipolar ions do not move toward either electrode. If the dissolved acids are basic they will of course migrate toward the cathode, since they have a net positive charge, while the dicarboxylic acids will be attracted to the anode because of their net negative charge.

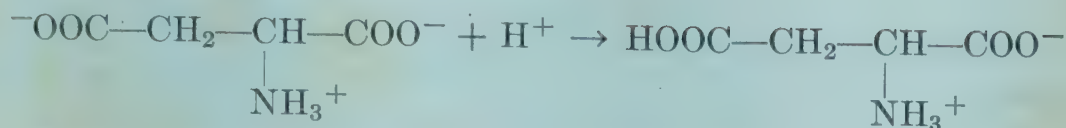


These facts are made use of in the process known as *electrophoresis*. By passing an electric current through a solution of amino acids they may be separated into the three fundamental classes, one group being found near the cathode, another near the anode, and the third still in the center compartment of the apparatus. This process will be referred to again in connection with protein analysis.

Although the neutral amino acids are the only ones whose net charge is zero when they are dissolved in pure water, it is possible to find for every amino acid a *pH* at which it will fail to migrate toward either electrode. If hydroxyl ions are added to the solution of a diamino monocarboxylic acid, they compete successfully for the protons on the amino groups and a neutral dipolar ion results.

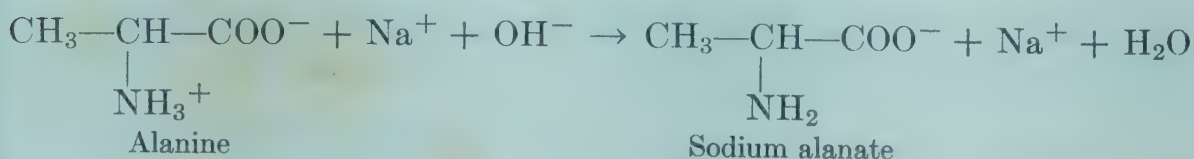


Similarly added hydrogen ion suppresses the ionization of the second carboxyl group in a dicarboxylic amino acid and again a neutral dipolar ion is formed.

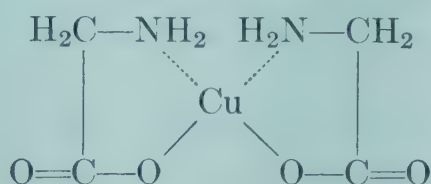


Thus, for each amino acid there is a definite *pH* at which it fails to migrate toward either electrode. This *pH* is called the *isoelectric point* of the acid. For the neutral acids this *pH* is in the vicinity of *pH* 6; for the acidic ones it is well on the acid side of neutrality while the basic amino acids carry a net charge of zero when the solution has some definite *pH* above 7.

3. *Reactions of the Carboxyl Group:* a. Salt Formation. As indicated above, the amino acids react with strong bases to form salts.

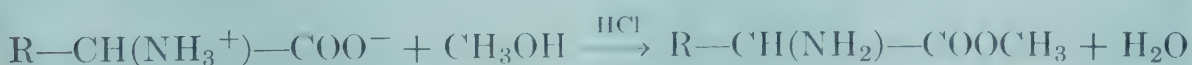


The amino acids also form a more complex type of salt. If a suspension of cupric hydroxide is added to a solution of a monobasic α - or β -amino acid, it dissolves with formation of a complex copper salt in which two amino acid residues are united through copper. These compounds are of the *ammine* type in which both the primary and the secondary valences of copper are involved, yielding a coördination compound. For such a glycine salt the following structure has been suggested:



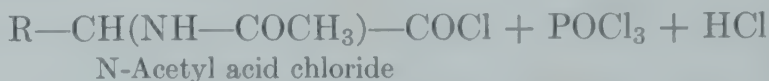
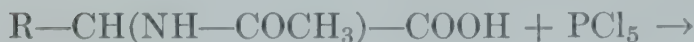
If cupric hydroxide is added to a solution containing a mixture of amino acids, the complex may hold together two different amino acids.

b. *Esterification.* Esterification of amino acids was first carried out by Emil Fischer as a step toward the separation of individual acids from a protein hydrolyzate. This separation is rendered very difficult by the great similarity of the amino acids and by the fact that they are not volatile and cannot, therefore, be separated by distillation. Fischer was able to esterify the mixture by heating it with alcohol saturated with hydrogen chloride, and he found that the resulting esters were low-boiling liquids. By fractional distillation at diminished pressure he isolated some of the previously known amino acids and discovered hydroxyproline.



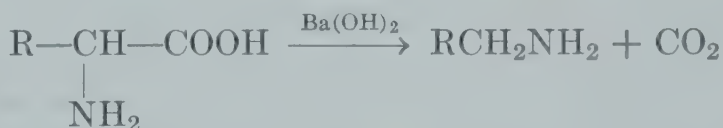
c. *Acid Chloride Formation.* Because of the adjacent amino group, it is difficult to form the acid chloride directly from an amino acid. These

compounds may be prepared however if the amino group is first protected by acetylation. Thus addition of acetyl chloride and phosphorus pentachloride to an amino acid brings about simultaneous acetylation of the amino group and formation of the acid chloride.



The protected molecule which is formed at this stage is often used in synthesis, or the protecting group may be removed by gentle hydrolysis, yielding the free acid chloride.

d. Decarboxylation. When amino acids are heated, especially in the presence of a weak base like barium hydroxide, many of them decompose with evolution of carbon dioxide.



A similar reaction takes place in the presence of certain putrefactive organisms. Some of the primary amines which result are toxic, and their formation from amino acids in the lower intestines is probably one cause of autointoxication.

4. *Reactions of the Amino Group:* With the exception of the prolines, the amino acids undergo all the reactions characteristic of the primary amino group.

a. Reaction with Nitrous Acid. This reaction, in which nitrogen is set free, is the basis of the Van Slyke¹ method for estimation of free primary amino groups (see p. 202).

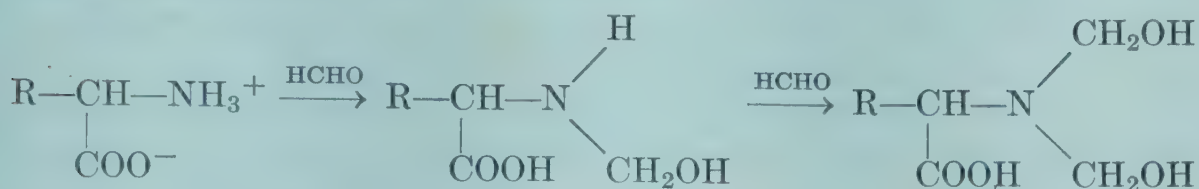


The nitrogen gas is collected and the equivalents of free amino nitrogen are calculated from the weight of nitrogen evolved. It should be noted that only half of the nitrogen actually collected comes from the amino acid.

b. Reaction with Formaldehyde. Formaldehyde reacts with the primary amino group in such a way as to eliminate that center of positive charge. There is some question about the actual structure of the derivative which

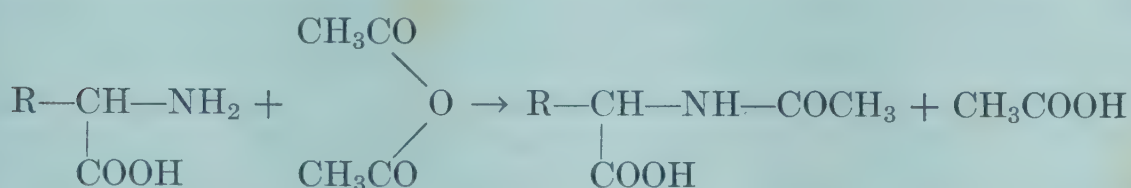
¹ Dr. Donald D. Van Slyke was for many years at the Rockefeller Institute for Medical Research in New York and is now Physiologist at the Brookhaven National Laboratory. He is known especially for his work on the acid-base balance in blood and for a number of excellent microanalytical methods.

forms, but with some acids at least the reaction seems to take place in two steps as follows:

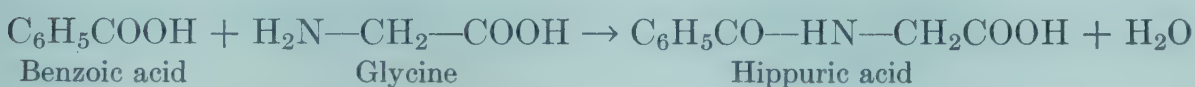


When the basic quality of the amino group has been suppressed in this way it is possible to titrate the carboxyl group as in any acid-base titration. This is the basis for Sørensen's "formol" titration method for estimation of free carboxyl groups.

c. Acetylation. As suggested above, the amino group reacts characteristically with acetylating agents such as acetyl chloride or acetic anhydride, forming an N-acetyl amino acid.



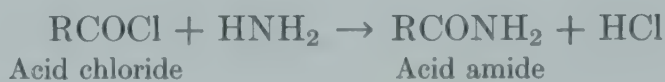
A similar reaction takes place in the body when glycine is conjugated with the toxic benzoic acid to yield the nontoxic product, hippuric acid, or N-benzoyl glycine.



d. Reaction with 1-Fluoro-2,4-dinitrobenzene. A reaction which has recently come into use in studies of protein structure (see p. 186) is the one in which a free amino group reacts with 2,4-dinitrofluorobenzene. The substituted amino acid which forms is colored yellow and is stable enough to withstand mild hydrolysis.

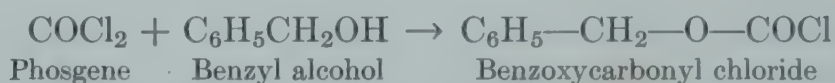


5. *The Linking of Amino Acids; Polypeptides:* The fundamental problem in amino acid chemistry, at least for the biochemist, is the manner of their combination in the complex protein molecule. From the beginning of his interest in proteins, Emil Fischer addressed himself to this problem. Side by side with his work on protein analysis, he carried out experiments designed to link several amino acids into a single molecule. His methods are now of historical interest only, but modern techniques must still find ways to block or protect the amino group. Once this has been achieved, an acid halide may be prepared and used to form a peptide bond ($-\text{CO}-\text{NH}-$) by a reaction resembling that by which a simple acid amide is prepared.

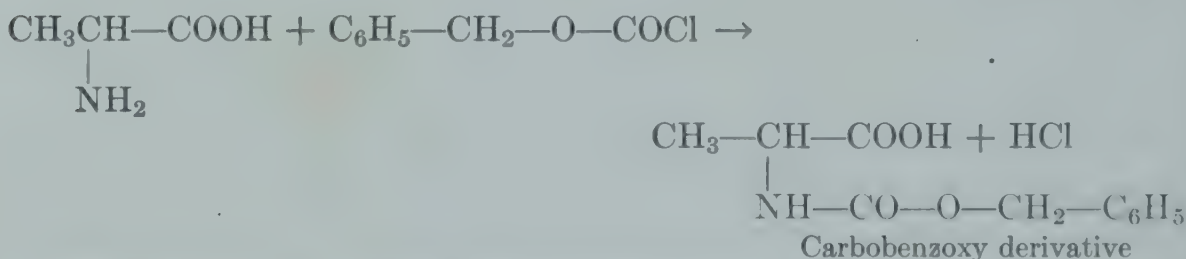


The compounds formed by linking of amino acids are known as di- or tri- or polypeptides, depending upon the number of amino acids bound.

a. Through the Carbobenzoxy Derivative. About 1932 Bergmann² introduced a method of protecting the amino group which revolutionized the preparation of peptides. We have already seen that an acid chloride may be prepared if the amino group is protected by acetylation. But this is of no use in the synthesis of peptides, because the acid hydrolysis which must later be used to remove the protecting group also splits the peptide bond. Bergmann introduced the use of a protecting group which may be removed by reduction instead of by hydrolysis. The reagent is prepared by interaction of phosgene with benzyl alcohol:

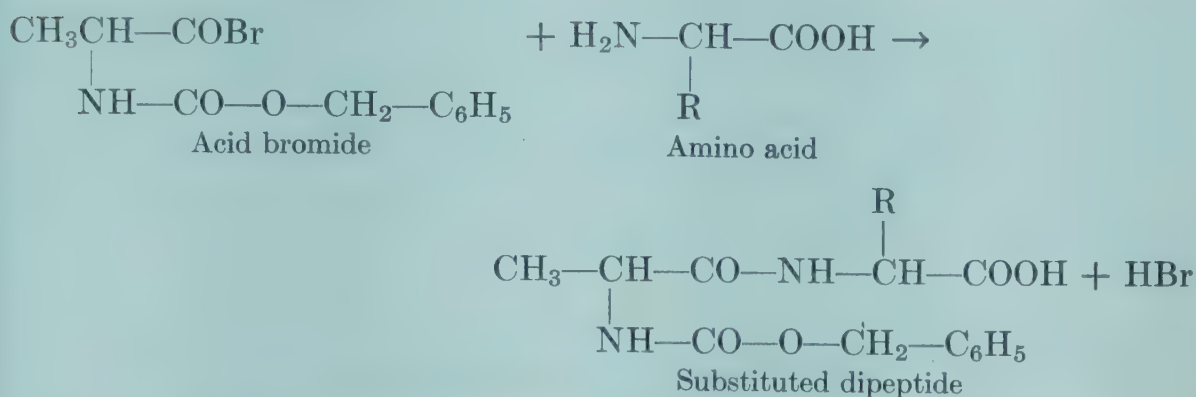


This reagent reacts with amino acids to block the primary amino group as follows:

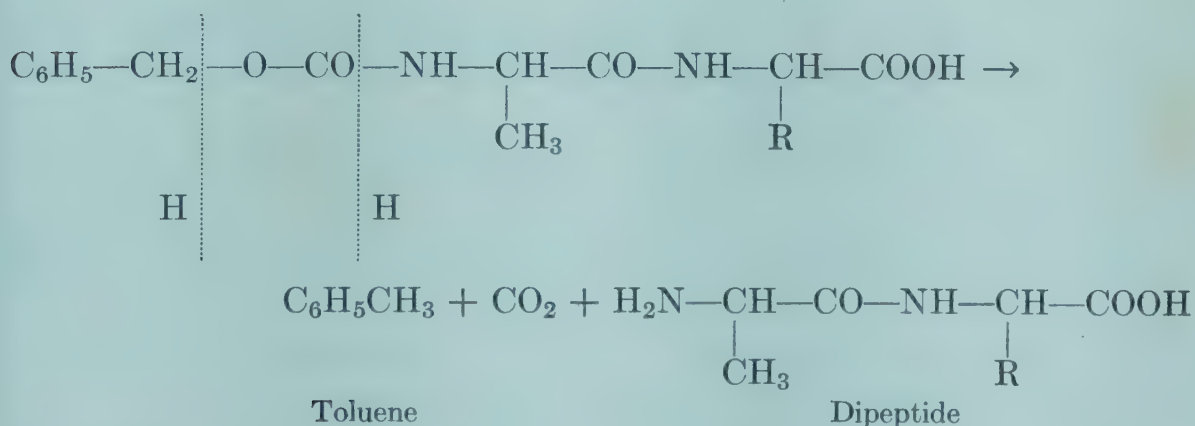


² Dr. Max Bergmann (1886-1944) became interested in amino acid chemistry while he was a student in Emil Fischer's laboratory. His research in the field of protein chemistry and structure was carried on first in Dresden, and after 1933 at the Rockefeller Institute in New York.

The protected derivative may now be treated as an acid. It forms an acid chloride or bromide which will condense with an amino acid to give a substituted dipeptide.



To free the dipeptide it is only necessary to reduce with hydrogen in the presence of colloidal platinum. The protecting group is split out with formation of toluene and carbon dioxide, and the dipeptide is set free.



Alternatively, the substituted dipeptide may be transformed into an acid halide and linked to other amino acids before removal of the protecting group.

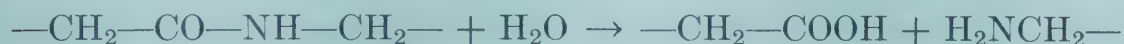
b. Through the Phthalyl Derivative: Although the Bergmann method made it possible to prepare a wide variety of peptides, it proved to have some disadvantages, particularly when the sulfur-containing amino acids were to be used. These disadvantages have been circumvented in a very recent synthetic method which makes use of the phthalyl group to mask the amino group during the actual coupling. Removal of the protecting group from the phthalyl dipeptide is brought about by reaction with hydrazine (N_2H_4), a reagent to which the peptide bond is insensitive under the specified experimental conditions. The preparation of the hydrochloride of glycylglycine by this method is illustrated by the following equations adapted from Sheehan and Frank's paper:

The bonds holding the elements in the backbone are believed to lie in a single plane, and this means that the R-groups project alternately above and below this plane.

The name "polypeptide" is given not only to synthetic substances of known structure, such as those just referred to. If a protein is hydrolyzed, it goes through a number of stages before it is entirely broken down to amino acids. With care the process may be interrupted at the stage at which the molecules are about the size of those formed when eighteen to twenty amino acids have been linked together to form a synthetic polypeptide. Such a solution contains what may be called "natural" polypeptides.

The justification for the name is found in a comparison of the properties of the natural and the synthetic substances. They are alike in solubility and both respond positively to the general protein color reaction known as the biuret test. This is dependent upon the presence in a molecule of at least two peptide bonds, separated by no more than a single carbon or nitrogen atom. The test is positive, for example, with biuret itself, $\text{NH}_2\text{—CO—NH—CO—NH}_2$.

Both the natural and the synthetic polypeptides can be hydrolyzed by heating with acids, or by the use of digestive enzymes. As the hydrolysis progresses, the number of free carboxyl and amino groups in each digest increases, and at approximately the same rate. This is of course to be expected of the synthetic substance since hydrolysis of such a compound must rupture peptide bonds and set free carboxyl and amino groups in equal numbers.



But the fact that the hydrolysis evidently progresses in much the same way with the natural products points to a similarity of the linkages in the two compounds.

Far more striking than this, however, is the fact that both compounds are attacked by the same digestive enzymes. One of the most characteristic properties of those natural catalysts is their extreme specificity. An enzyme designed to bring about the hydrolysis of sucrose will not attack lactose. Even a change in optical rotation about a single carbon may render a molecule inert toward a particular enzyme. It is therefore extremely significant that enzymes which digest natural proteins, also hydrolyze the synthetic polypeptides.

From these facts it was concluded long ago that the amino acids in proteins are probably linked, at least in part, through peptide bonds. Later work has confirmed and extended this conclusion so that it is now recognized that these bonds constitute the chief linkages in the protein mole-

cule. This conclusion has recently been confirmed by infrared spectroscopy which clearly reveals the presence in proteins of simple amide linkages.

THE PROTEINS

Having now learned something of the chemical properties of the individual amino acids we are ready to consider their condensation products, the proteins.

Isolation of Proteins from Tissues. Many proteins occur in solution in natural fluids such as blood serum or digestive juices. Others may be extracted from tissues by the use of such solvents as dilute acid or base or sodium chloride solution. Either solution will contain several different proteins with nearly identical properties as well as a number of small organic and inorganic molecules. The isolation of a single protein from such a mixture is a difficult matter, and all that can be done here is to indicate some of the methods by which it may be attempted.

It has already been indicated that certain *protein precipitants* can be counted upon to throw out of solution almost all proteins. Alcohol in sufficient concentration is such a general precipitant, and the alkaloidal reagents also remove protein from solution, presumably by forming such salts as protein picrate or phosphotungstate. Such a procedure serves to separate the extracted protein from soluble nonprotein material. Sometimes it is possible even at this stage to make a partial separation of the proteins themselves. Some proteins precipitate if their solution is partially saturated with such a neutral salt as ammonium or magnesium sulfate. Others separate only from the completely saturated solutions. The "globulin" extracted from fat-free peanut meal is divided into two fractions if its solution is first made 0.2 saturated with respect to ammonium sulfate. This throws down part of the dissolved protein, which may be separated from the solution by filtration, or better by use of a centrifuge. Complete saturation of the filtrate "salts out" a second protein fraction.

Some proteins precipitate when their solution is brought to a definite acidity. Like the amino acids, the proteins have an *isoelectric point* which depends upon the nature of the constituent amino acids and upon the number and nature of the links between them. At some definite pH value the net charge on the molecule is zero, and at this pH certain physical properties of protein solutions have a maximum or minimum value. Viscosity, osmotic pressure, and solubility in water, for example, are at minimum values at the isoelectric point. Some proteins precipitate directly if the pH of the solution is brought to this critical value. Casein, the chief protein of milk, is thus separated from other proteins when milk is brought to pH 4.6.

Dialysis is frequently employed in the process of isolating proteins. Sometimes a crude protein extract is dialyzed against running water to

separate it from small molecules which have been extracted with the protein and which pass through the membrane and are carried away. In other procedures a protein precipitate, obtained by salting out, may be suspended in water and the suspension dialyzed against water. As the small salt molecules which brought about the precipitation pass into the water the protein goes back into solution. With proteins which cannot be crystallized this process of alternate precipitation and dissolution may be repeated again and again to effect a partial purification.

The *ultracentrifuge* has proved to be an instrument of great value both in purification of proteins and in assessing the purity of a supposedly homogeneous sample. As previously noted, these instruments are capable of speeds up to 80,000 revolutions per minute and develop a centrifugal force equivalent to more than 400,000 times gravity. When a protein mixture is subjected to such a force as this, any molecules which are conspicuously heavier than others go quickly to the bottom, leaving the lighter molecules suspended in the liquid. Thus when skimmed milk is spun in the ultracentrifuge its casein separates readily, leaving behind in the "milk serum" a number of protein constituents of lower molecular weights.

Criteria of Purity of Proteins. Some proteins remain amorphous through repeated precipitations and dissolutions but are by such procedures separated into fractions which become progressively more homogeneous. Others separate as crystals quite readily. But not even with the crystalline proteins is it a simple matter to determine when a sample is pure. None of the usual criteria for the purity of organic compounds can be applied. The proteins do not melt but decompose at elevated temperatures. No distinction between fractions can be made on the basis of elementary analyses since the elementary analysis of one protein is very like that of another. Fortunately, however, there are now available physical methods which not only facilitate the isolation of proteins but help to assess the purity of the final sample.

We have already seen how the ultracentrifuge may be used to separate proteins of different molecular weights. Many supposedly pure proteins have been shown by this technique to consist of a mixture of several very similar components. The behavior of such a mixture in the ultracentrifuge is graphically illustrated in Figure 5.1. Skimmed milk was subjected for varying lengths of time to a high centrifugal force and the concentration of protein at different levels in the tube was determined by a physical method. In the graphs the ordinates give the protein concentration in arbitrary units, plotted against the distance in centimeters from the center of rotation. Each peak corresponds to molecules of a certain weight, and the area under a peak is proportional to the total concentration of those molecules. The nearer a peak is to the right side of the diagram, i.e., the farther from the center of rotation, the heavier are the molecules responsible for the peak.

Diagram A shows the distribution of seven different peaks after 13 minutes' spinning at full speed and indicates their relative concentrations. In Diagram B, as a result of 49 minutes' spinning, the heavier molecules have moved beyond the 7 cm. line and the lighter ones have spread out more widely. Diagram C shows the beginning of the separation of two components from the first peak, and in Diagram D these two have become distinct as a result of 185 minutes' centrifugation at full speed.

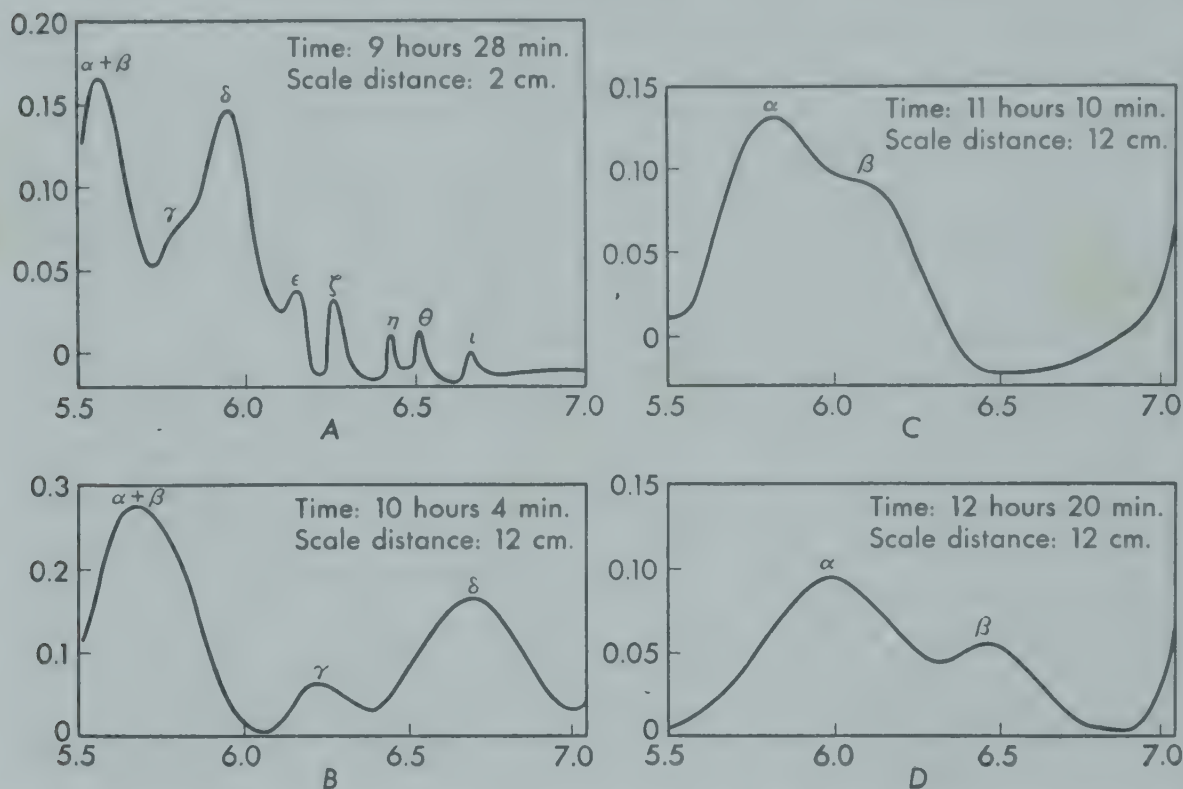


Figure 5.1. Ultracentrifugal sedimentation diagrams of skim milk which had been previously dialyzed for several days against buffer solutions. The abscissae give in centimeters the distances from the center of rotation and the ordinates give measurements which are proportional to the concentration of protein at that distance. The peaks correspond to molecules of a certain size. The faster a peak moves to the right, the larger the molecules. Centrifugal force: 260,000 times gravity. (From K. O. Pedersen, *B.J.*, 30:956, 1936.)

A quite different picture is obtained if the protein solution is homogeneous. Figure 5.2 shows the way in which a single peak moves along the tube with time, showing no emergence of secondary peaks. When long exposure of a protein solution to a high centrifugal force gives no evidence of a second component, the solute has passed one crucial purity test.

The electrophoresis technique, originated by Tiselius,³ offers another method both for fractionation of protein mixtures and for examining the purity of a supposedly homogeneous sample. This method makes use of the fact that proteins in neutral salt solution carry an effective net charge which may be negative or positive, depending upon the isoelectric point

³ Arne Tiselius, Professor of Biochemistry at the University of Upsala, Sweden, brings to biochemical problems the point of view of a physical chemist.

of the protein. This depends in turn upon its amino acid composition. A high percentage of dicarboxylic acids will make a protein essentially anionic, that is, it will exist as a hydrogen or metallic proteinate. If the diamino, monocarboxylic acids predominate, the protein will be present in solution largely as protein chloride, for example, and will carry a positive charge. When a mixed protein solution is subjected to a constant electric current, the charged protein particles move toward the electrode of opposite charge.

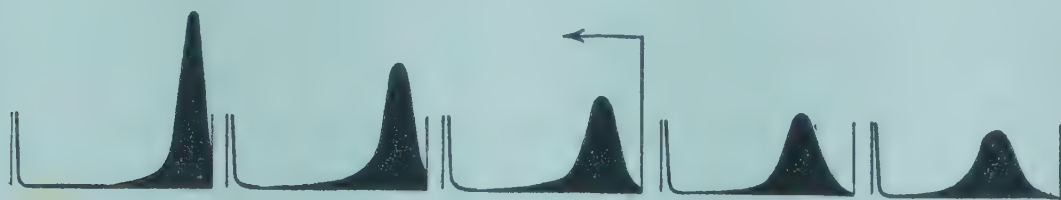


Figure 5.2. Sedimentation diagram of a solution of crystalline growth hormone in glycine buffer. The first picture on the left was taken 20 minutes after attaining full speed; subsequent photographs were made at 16-minute intervals. The arrow indicates the direction of sedimentation from the meniscus. Speed = 59,780 R.P.M. (From E. L. Smith, D. M. Brown, J. B. Fishman, and A. E. Wilhelmi, *J.B.C.*, 177:307, 1949.)

The exact form of apparatus used for such a separation will depend upon the purpose of the experiment. Apparatus designed for the separation of protein mixtures uses a large "cell" divided in some way into separate compartments. Tiselius designed such a cell with twelve separate compartments separated by sheets of parchment paper near the cathode, with leather near the anode and with cotton flannel in intermediate sections. Figure 5.3 shows a simpler cell which is divided by sheets of sintered glass

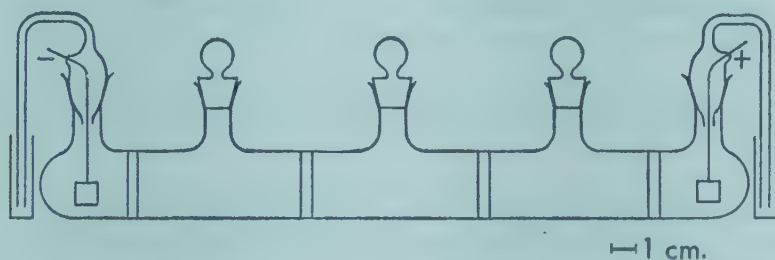


Figure 5.3. An electrophoresis cell in which the compartments are separated by sintered glass disks. Capacity of Cells I and V, 40 ml.; Cells II, III, and IV, 80 ml.; platinum electrodes. (From G. W. Irving, H. M. Dyer, and V. duVigneaud, *J.A.C.S.*, 63:505, 1941.)

into five compartments. When a protein solution is put into the center compartment and electrolyte solution in the others and the whole is subjected to an electric field, the charged particles move toward anode or cathode through the glass, but at the end of the experiment the solution from each compartment can be removed separately for further study. In one such experiment with a previously purified protein solution it was found that nearly 70 per cent of the protein had concentrated in the cell nearest the cathode compartment.

When the electrophoresis is to be carried out, not for the purpose of fractionation, but only to determine the purity of a small sample, the smaller cell illustrated in Figure 5.4 may be used. The rate and direction of migration of the boundary between the protein and supernatant buffer solution are determined by one of several physical methods. If but one boundary appears, moving smoothly toward a single electrode, the solution is said to be electrophoretically homogeneous. If such a substance appears also to be homogeneous in the ultracentrifuge its purity is as nearly

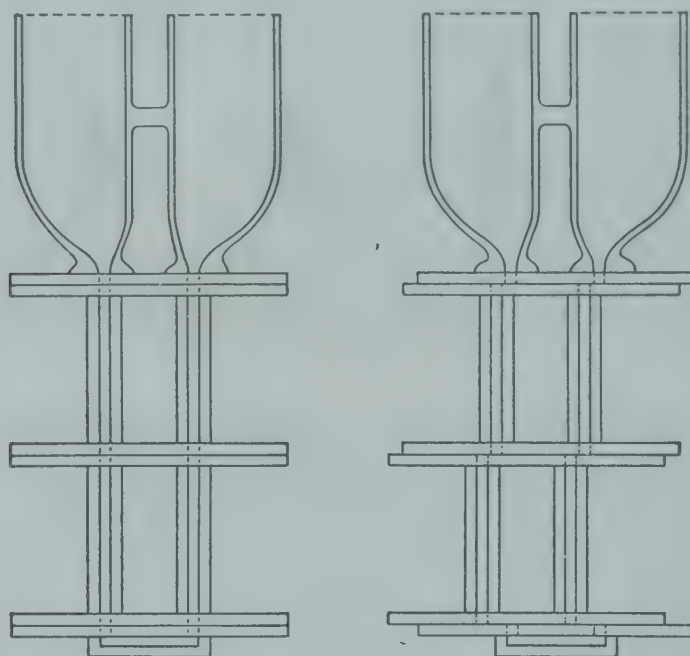


Figure 5.4. The U-tube used in the Tiselius electrophoresis apparatus for observing the migration of charged particles. Note that in this form of cell the solution can be separated, after electrophoresis, into several fractions by displacement of the sections as shown in the diagram on the right. (From A. Tiselius, *The Harvey Lectures*, 35:37, 1939-40.)

proved as is possible at present and it is considered to be a single chemical entity.

How complex such an entity is and how inadequate are our chemical and physical tests of purity become clear when the immunological reactions of proteins are considered. A protein of moderate molecular weight contains about three hundred amino acid residues,⁴ representing about twenty different amino acids. One needs only an elementary understanding of permutations and combinations to realize that the number which represents all possible arrangements of these residues is so large as to be quite inconceivable. It should therefore be clear that proteins might be elaborated in living tissues, identical in all measurable physical and chemical

⁴ An acid "residue" is that part of the acid which is represented in a polypeptide chain. A serine residue, for example, consists of —HN—CH—CO— .



properties, and yet not identical in their ultimate molecular architecture. They might differ, for example, in the actual number of different acids present, or in their ratio to each other or in the order of their arrangement in the polypeptide chain. This type of difference between proteins can best be detected serologically.

When a protein from one species of animal is injected into another species it acts as a foreign substance or antigen and gives rise in the blood of the host to antibodies which are themselves protein in nature. The flocculation which results when serum of the host is mixed with a solution of the antigen is believed to depend upon a specific structural relationship between the two proteins. It is therefore of great interest that antibodies formed in response to serum albumin of one species do not precipitate in the presence of serum albumin of another species. This must indicate fine structural differences between proteins which respond identically to the ordinary chemical tests. Apparently under the mediation of cell catalysts, each species uses the twenty-one or twenty-two fundamental amino acids to build highly individual and specific protein molecules, each generation reproducing faithfully its own ancestral pattern.

In spite of the difficulties which have just been indicated, a few proteins have been prepared in apparently pure form. Even when absolute purity has not been achieved it has been possible to carry out many satisfactory studies of protein chemistry with samples which approach homogeneity. Indeed much of our present information about proteins has been obtained by the use of partially purified substances.

Classification of Proteins. The proteins were classified near the turn of the century on a purely empirical basis, which depended chiefly on differences in solubility. No fundamental revision of this classification has been made in the intervening years. It was never very satisfactory but does separate a large number of very similar compounds into classes which are useful in separation of mixtures. A real classification must await a much more detailed knowledge of the chemistry of the proteins.

All proteins may be roughly divided into three main groups as follows:

A. *Simple proteins* are compounds which occur in nature and yield on hydrolysis only α -amino acids or their derivatives.

B. *Conjugated proteins* also occur naturally but when they are hydrolyzed they set free in addition to α -amino acids various nonprotein molecules, such as carbohydrate or hemin. The nonprotein part of such a molecule is known as a "prosthetic group."

C. *Derived proteins*, as the name implies, are substances formed from native proteins. The molecules are still of colloidal size but may be formed by partial hydrolysis or by other slight modification of the natural protein.

These main classes are further subdivided as follows:

A. *Simple Proteins.*

Albumins. Soluble in water and in neutral salt solutions; coagulable by heat.

Examples are serum albumin from blood and ovalbumin from white of egg.

Globulins. Insoluble in water but soluble in dilute solutions of neutral salts; coagulable by heat; insoluble in saturated solutions of neutral salts or in half-saturated ammonium sulfate. Examples are serum globulin and edestin from hemp seed.

Glutelins. Insoluble in water or neutral salt solutions but soluble in dilute acid or alkali. An example is glutenin from wheat. This type of protein has been found only in plants.

Prolamines. These are also plant proteins. Their solubility is like that of the glutelins but they differ in being soluble in 75 per cent alcohol. Contain a high percentage of proline. An example is gliadin from wheat.

Albuminoids (Scleroproteins). Extremely insoluble except in reagents which bring about decomposition. Examples are keratin from wool and fibroin from silk.

Histones. Soluble in water and in dilute acids or strong bases; weakly basic in character due to a high percentage of diamino acids. An example is globin from hemoglobin.

Protamines. Soluble in water and in dilute acids; not coagulable by heat; composed largely of diamino acids so that their aqueous solution is basic to litmus; occur chiefly in combination with nucleic acid; the molecules are smaller than most proteins, and so diffuse through collodion. An example is salmin from salmon sperm.

B. *Compound or Conjugated Proteins.*

Nucleoproteins. The prosthetic group is nucleic acid, a complex substance of high molecular weight containing both nitrogen and phosphorus.

Chromoproteins. Compounds of protein with some colored molecule which contains a metal. Examples are hemoglobin from which may be set free the iron-containing substance, heme, and hemocyanin from invertebrate blood. The latter consists of a protein moiety linked to a blue, copper-containing molecule.

Phosphoproteins. According to British classification these are simple proteins, though hydrolysis frees phosphoric acid which is neither combined in a phospholipid nor in nucleic acid in the original molecule. An example is casein from milk.

Glycoproteins. The prosthetic group is carbohydrate in nature. Mucin from saliva is an example.

It is possible that there are other conjugated proteins but they have not yet been well characterized.

C. *Derived Proteins.*

Primary Protein Derivatives. These are substances derived from proteins by changes less drastic than hydrolysis. *Denatured proteins*, representing the least possible change in the protein molecule, belong in this group. Nonhydrolytic

changes brought about by brief exposure to acids or alkalis result in *metaproteins*.

Coagulated proteins are also considered primary derivatives.

Secondary Protein Derivatives. While still having molecules large enough to exhibit many of the properties of proteins, these are early products of protein hydrolysis. Hydrolysis of proteins does not, as might have been expected, proceed by a splitting of amino acids one by one from the edges of the molecule. Rather, it begins with a splitting of the huge molecule into two or three roughly similar fragments. As this process is repeated the molecules finally become too small to be considered proteins any longer. The gradual change in molecular size is reflected in changes in coagulability and in solubility. On this basis the hydrolysis of proteins is believed to go through the following stages:

Proteoses. Soluble in water and not coagulated by heat. Precipitate from saturated ammonium sulfate solution.

Peptones. Water-soluble and not coagulable by heat. Not precipitated by saturated ammonium sulfate but are precipitated by some alkaloidal reagents.

Peptides. This group includes all the smaller fragments which are not precipitated by any of the "protein reagents." The largest of these are called "polypeptides" and are degraded ultimately to tri- and dipeptides and amino acids.

Quantitative Analysis of Proteins. To obtain information about the amino acids present in a protein it must be hydrolyzed completely, and its constituent amino acids must be determined quantitatively. Emil Fischer devised the first satisfactory separation when he transformed into esters the mixture of acids obtained by hydrolysis of protein and separated these esters by fractional distillation under diminished pressure (see p. 159). This procedure however is more satisfactory for isolation from a hydrolyzed protein of those amino acids which are present in high proportion than for identifying all of the amino acids present. The method requires at least 100 g. of protein and never yields even approximately complete analytical figures.

Modern methods have improved upon Fischer's separation in various ways. After hydrolysis of the protein is complete, the mixture of amino acids is usually subjected to a preliminary fractionation. For example, a separation into three fractions may be achieved by use of one of the large electrophoresis⁵ cells. The basic amino acids concentrate near the cathode; the dicarboxylic acids move toward the anode compartment, while the large group of neutral acids remains in the central part of the cell. It is for such a separation that the cell shown in Figure 5.3 is used.

⁵ For the separation by means of the electric current of substances with different net charges, the words "electrophoresis," "ionophoresis," and "electrodialysis" are used interchangeably. It has been suggested that the first be used for separation of large molecules; that ionophoresis be used when small molecules are involved, while electrodialysis be used to designate the separation of large from small molecules by simultaneous use of a semipermeable membrane and the electric current.

Following such a preliminary separation, the concentration of the individual acids may be determined in different ways. Some are precipitated by specific reagents. Histidine, for example, may be separated from the other basic acids by precipitation as the insoluble silver salt. Other acids lend themselves to quantitative colorimetric estimation without preliminary separation from a mixture. For example, the characteristic color of arginine with the Sakaguchi reagent (see p. 205) may be used to determine its concentration. Several other acids with functional groups other than the amino and carboxyl groups may be estimated colorimetrically, but for a complete analysis it is necessary to turn to other methods.

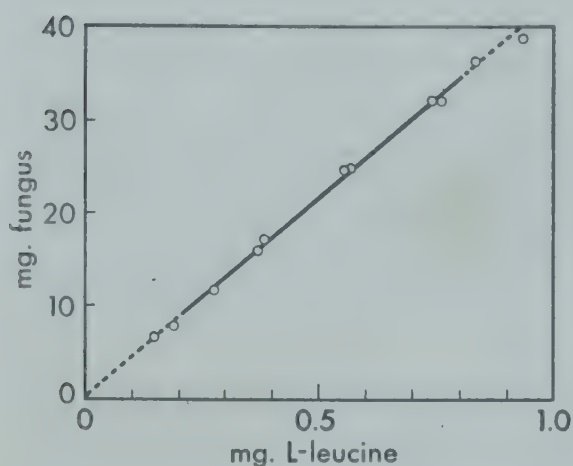


Figure 5.5. The relation between the weight of leucine added to the medium and the weight of fungus which has formed at the end of 8½ days of incubation. (From F. J. Ryan and E. Brand, *J.B.C.*, 154:167, 1944.)

Microbiological Assay: One of the most elegant analytical methods is the procedure known as "bio-assay," which requires no preliminary isolation of the acids. It depends upon the fact that microorganisms can be bred so that a certain strain requires in the nutrient medium a specific amino acid. If a culture of an organism requiring histidine, for example, is transferred to medium lacking this necessary amino acid, it fails to grow at all. If a little of the essential acid is present, growth proceeds at a sub-optimal rate; if an adequate amount is present, growth is satisfactory. By the use of pure amino acids it is possible to determine the exact growth rate for a particular strain in the presence of different amounts of the essential acid. Figure 5.5 shows the result of adding graded amounts of natural leucine to small inoculations of one strain of the fungus, *neurospora*. Clearly the dry weight of fungus obtained is directly proportional over this range to the amount of leucine available to the organism. If now the experiment is repeated, using in the nutrient solution in place of the pure leucine, graded amounts of a protein hydrolysate, it is a simple matter to compute from the growth curve exactly the amount of leucine which must have been present in the original protein. This assumes, of course, that the medium contains plenty of all other dietary essentials. Even though such experiments involve incubation for eight to ten days, they are simple compared with the elaborate chemical manipulations which are required

for actual quantitative separation of the acids. Furthermore, the method of bio-assay requires comparatively small amounts of protein. It has therefore been adapted to the estimation of one amino acid after another, and current journals still add to the strains of microorganisms suitable for such testing. All the acids known to be protein constituents can now be estimated by one or another of the microbiological methods.

Chromatographic Analysis: A method of estimation of amino acid concentration which does involve separation of the acids is a recent modification of a much older method. The name "chromatography," originally given to a process in which colored substances were separated from each other, is now applied to a wide number of adaptations of the original method.

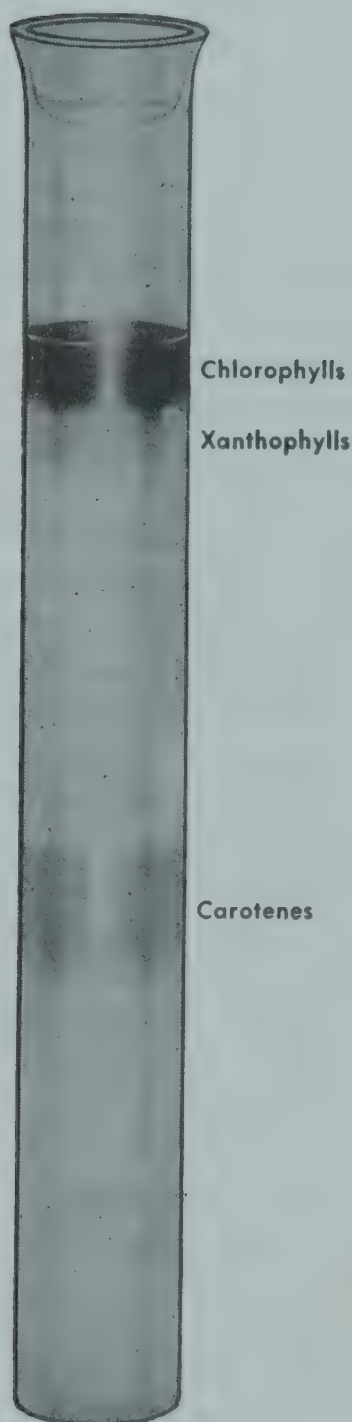
The various chromatographic processes depend upon a few basic facts: (1) Substances differ in the readiness with which they are adsorbed upon a surface. When an organic compound is freed of colored impurities by boiling its solution with decolorizing charcoal, the success of the separation depends upon the fact that the colored molecules are adsorbed on the surface of the carbon while the others remain in solution. (2) The relative ease of adsorption depends in some measure upon the solvent employed. This fact is made use of in enzyme chemistry where it is often possible to cause adsorption of an enzyme upon some colloidal material and then to remove, or elute, it by shaking the adsorbent-complex with a different solvent, or with the same solvent at a different pH.

These facts were used early in the century when an adsorption column was first elaborated for chlorophyll studies. But it was not until about 1931 that Kuhn⁶ and his co-workers made chromatographic analysis a common laboratory method. In its simplest form the method uses a long glass tube packed with an adsorbing material such as aluminum hydroxide or the clay known as "fuller's earth." If a solution containing a number of colored solutes is allowed to trickle down this column, the solutes are selectively adsorbed. The rate of migration of a given substance down the column is nearly inversely proportional to what is known as its *adsorption coefficient*. The adsorption coefficient is defined as the ratio of the amount adsorbed per gram of dry column material to the concentration of the solute in the solution. If its adsorption coefficient is high it is readily adsorbed, is difficult to elute and will remain near the top of the column. If its adsorption coefficient is low, it will travel down the column rapidly. After the solution has passed through the tube the chromatogram is "developed" by passing a large volume of the same or a different solvent slowly through the column. This results in innumerable elutions and re-adsorptions, with

⁶ Richard Kuhn was born in Vienna in 1900. For many years he published from Zürich, but he has been at the Kaiser-Wilhelm Institute for Medical Research in Heidelberg since 1928. He was awarded the Nobel Prize in Chemistry in 1938 but was not able to accept it.

each substance moving down the column at its own rate. Each will after a time occupy a zone which appears as a colored band in the white column. In Figure 5.6 is shown the separation of a crude cyclohexane extract of grass into three different colored components. The column was packed with magnesium oxide and the position of the chlorophylls near the top shows that they were strongly adsorbed. From such a column the three components could be recovered by pushing out the magnesium oxide cylinder and cutting out the colored portions.

Although the amino acids are colorless, the chromatographic method has been adapted very interestingly to their separation and estimation. The



list of possible adsorbents is now a long one. It includes such simple substances as activated (heated) carbon, specially prepared aluminum hydroxide, various natural clays, and even powdered sugar and starch. Of late years it has been found that various polymerized resins lend themselves well to such uses and act with great selectivity. If a solution of mixed amino acids is passed through a column packed with a poly-amine-formaldehyde resin known commercially as "Amberlite IR4," the dicarboxylic acids are preferentially adsorbed. Use of a series of columns containing various commercial preparations of the fuller's earth type will separate the basic acids one by one from each other and from all the remaining amino acids. One resin adsorbs tryptophan preferentially; a special preparation of aluminum oxide holds back cystine. From such columns the adsorbed amino acid is later recovered by elution with some solvent in which its adsorption coefficient is much smaller so that it leaves the solid and goes into solution.

An interesting version of the chromatographic method, developed by Stein and Moore at the Rockefeller Institute, makes it possible to determine the distribution of amino acids in as little as 2.5 mg. of protein. After hydrolysis of the protein the amino acid mixture is passed through an adsorbing column of starch or one of the resins. The development consists of a continuous, 24-hour

Figure 5.6. Separation of a grass extract dissolved in cyclohexane into three fractions by passing it through a column of magnesium oxide. (From A. J. P. Martin, *Endeavour*, 6:21, 1947.)

elution from the column by which the acids pass down the tube and are finally carried out in the solvent. The effluent solution is caught in separate 0.5 ml. fractions which are analyzed separately by measuring the

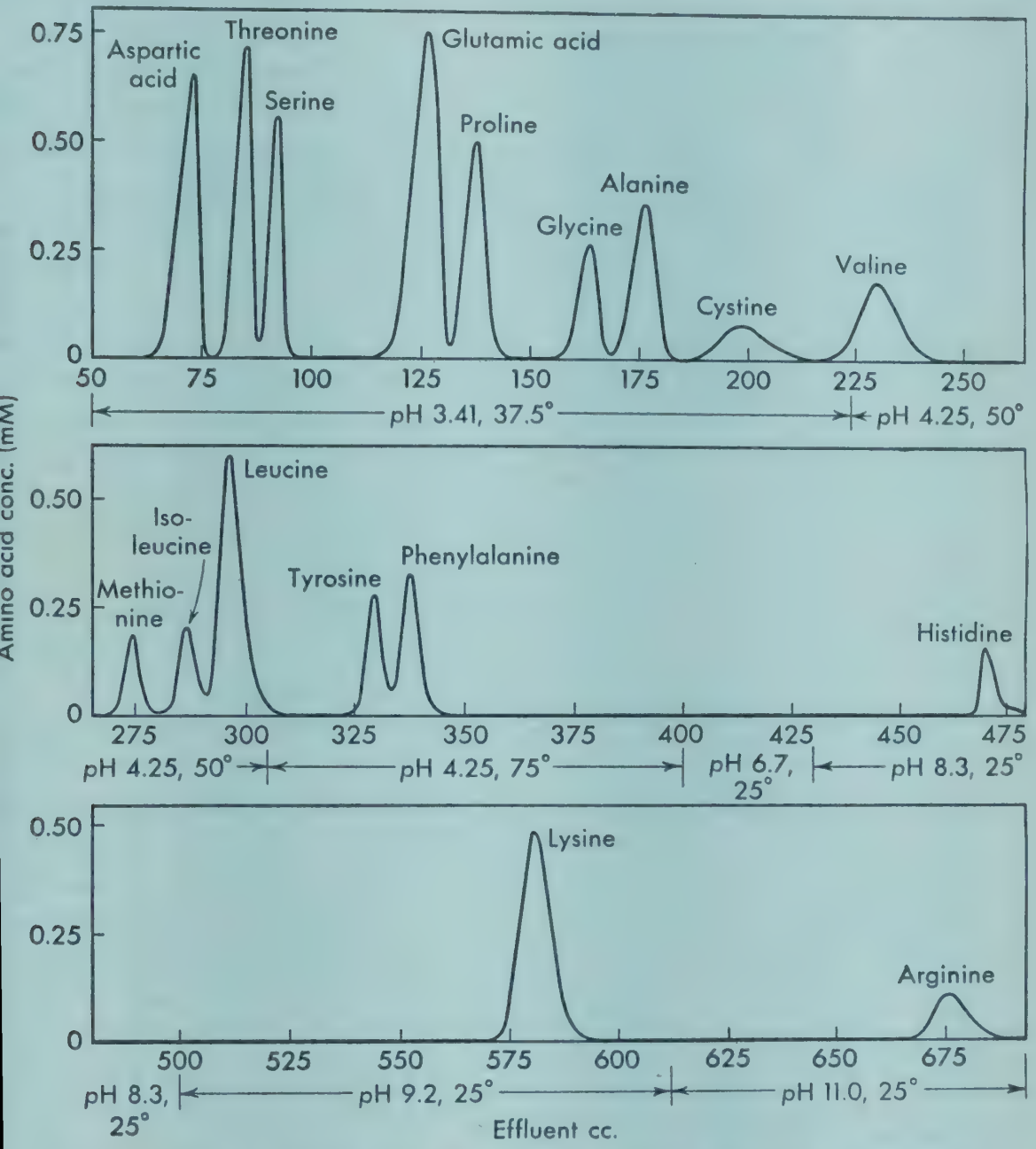


Figure 5.7. Separation of amino acids from a synthetic mixture simulating the composition of a protein hydrolysate. The column of Dowex-50, a synthetic resin, was used in the sodium form and the eluants were buffers as indicated on the graph. The original mixture weighed about 6 mg. The abscissae represent the ml. of effluent. Thus the first amino acid to appear was aspartic acid, while arginine was not recovered until 675 ml. of solvent had been collected. (From S. Moore and W. H. Stein, *J.B.C.*, 192:664, 1952.)

color developed with the ninhydrin reagent (see pp. 203 and 207). When the concentration of amino acid in each fraction is plotted against the number of milliliters of effluent which have run out, such a curve as that shown in Figure 5.7 is obtained. Each peak usually represents a separate amino acid and the area under the peak is proportional to the total concentration

of that acid. Occasionally a peak will include two or more acids and then this must be resolved by using a different solvent. The final graphs give an

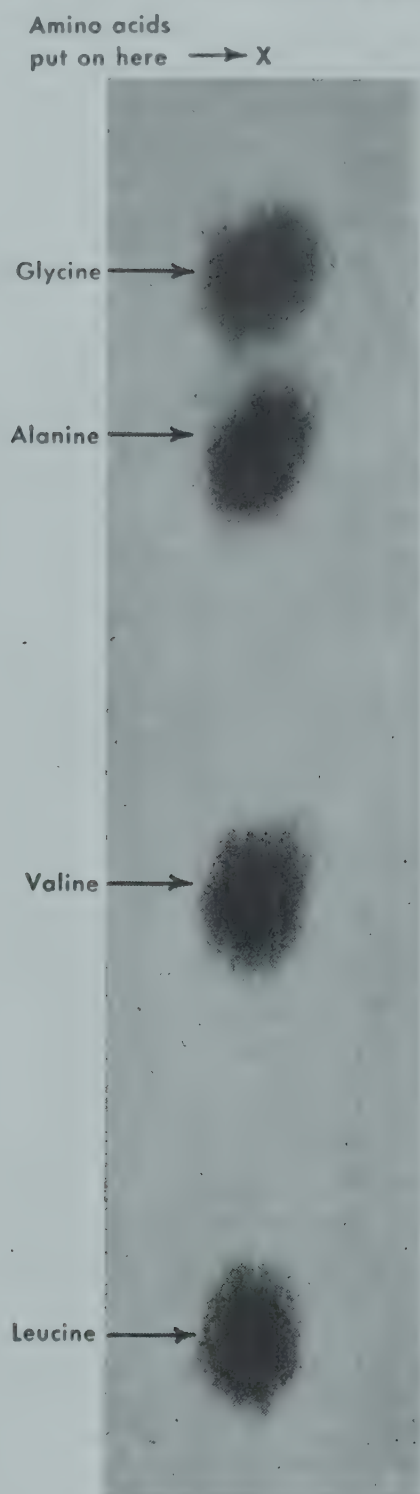


Figure 5.8. Separation of glycine, alanine, valine, and leucine by filter paper chromatography, using butanol as solvent. (From A. J. P. Martin, *Endeavour*, 6:25, 1947.)

accurate and complete picture of the amino acid content of the original mixture. This achievement should be assessed with two facts in mind. The older methods of protein analysis, incomplete as they were, required many grams of material instead of the few milligrams used by Stein and Moore. Emil Fischer's analysis began with at least 100 g. of protein. And even with such amounts, as recently as 1930 only about half of the known amino acids could be determined with any degree of accuracy. In two decades the methods of protein analysis have been completely revolutionized.

An offshoot of the chromatographic method which is now beginning to yield quantitative results is the one known as *paper partition chromatography*. This uses as adsorbent a sheet of filter paper saturated with one solvent, and allows a mixture of amino acids dissolved in a second solvent to pass slowly down the paper. It was originally believed that the inert paper simply served as a support for the first liquid, and that the process was one in which the rate of travel of the solute down the paper was determined simply by its distribution ratio in the two sol-

vents. This seems now not to be true. The phenomenon is apparently strictly comparable with the ones just discussed in which the adsorption coefficient is the important factor.

A strip of filter paper moistened, for example, with water constitutes the column. If a drop of a solution of mixed amino acids is put at the top of such a strip, and then a solvent is allowed to run slowly down the paper, each acid will move down at a rate determined by the nature of the two

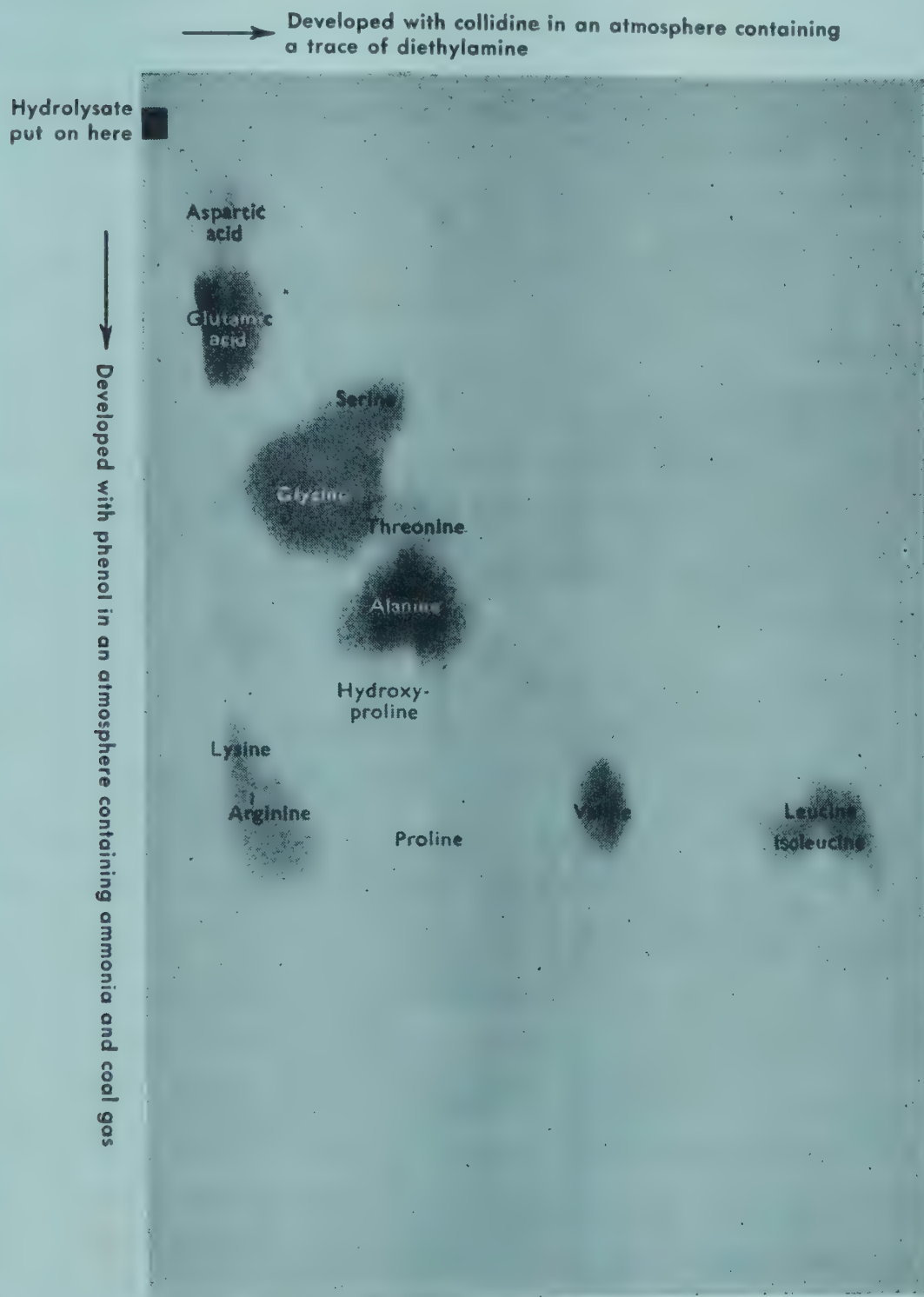


Figure 5.9. Two-dimensional chromatogram of a gelatin hydrolysate. A drop of the solution was placed at the corner of the paper as shown. The chromatogram was developed with *s*-collidine, dried, and then developed at right angles to the first procedure with phenol. After drying a second time the paper was sprayed with ninhydrin and warmed to bring out the color wherever an amino acid had accumulated. (From A. J. P. Martin, *Endeavour*, 6:26, 1947.)

liquids and by the adsorption coefficient of the individual acid. This method is improved if the paper is a large sheet instead of a strip. In this case, the mixed solution is put near one corner of the sheet. After development with one solvent, the paper is turned through 90 degrees and then developed with a second solvent which moves at right angles to the first. Figure 5.8 shows the way in which a mixture of four amino acids is spread out in a straight line during the running of a single solvent. A second solvent, used as indicated above, moves each acid a characteristic distance in a direction at right angles to that first traveled. Figure 5.9 shows the positions occupied by a number of amino acids when the chromatogram was developed first with collidine (2,4,6-trimethylpyridine) and then, at right angles, with phenol saturated with water. The spots form when the paper is heated with ninhydrin reagent. The distance an acid moves in the two directions is characteristic so that the relative positions of the acids in a chromatogram is fixed. Comparison of the spots obtained from a protein hydrolysate with those from a mixture of known acid content determines with astonishing accuracy the composition of the unknown solution.

The number of proteins whose amino acid content is accurately known has increased rapidly of late years. To indicate these results in small space, it is suggested that a protein analysis be reported in such a form as the following, using the first two or three letters of its name to stand for each amino acid:

PEPSIN, MW 34,400, Total N 14.60%

Gly₂₉Ala..Val₂₁Leu₂₇Ileu₂₈Pro₁₅Phe₉(CySH)₂(CyS-)₄Met₄Try₄

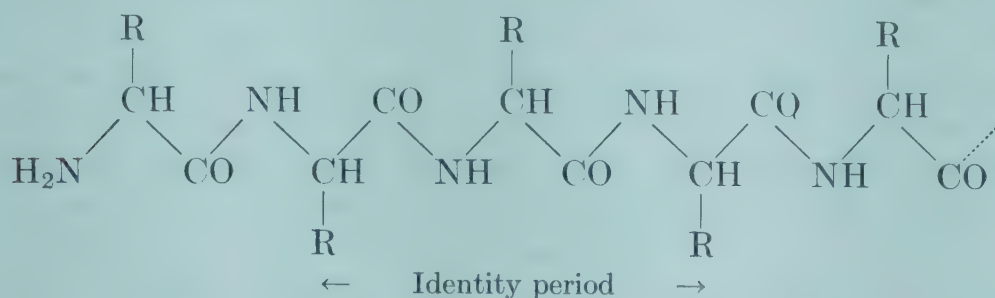
Arg₂His₂Lys₂Asp₉Glu₂₈(Asp-NH₂)₃₂Ser₄₀Thr₂₈Tyr₁₆(H₂O)₂(H₂PO₃)

The figures refer to the number of residues of the acid per molecule of protein. Asp stands for aspartic acid and Asp-NH₂ for its acid amide, asparagine, HOOC—CH(NH₂)—CH₂—CONH₂.

Structure of Proteins. It has long been realized that the fundamental linkage in the proteins is a peptide bond. The problem of protein structure then resolves itself into two questions: First, can anything be learned of the order in which the acids are linked? Second, how is this enormous polypeptide disposed in space; what is its actual shape? The ultracentrifuge gives a partial answer to the second question. Certain of the proteins have a sedimentation rate in the ultracentrifuge which points to their being simple polypeptide chains, many hundred times as long as they are thick. These include keratin or wool protein, fibroin from silk, and myosin from muscle, the so-called *fibrous* proteins. Others, and these include most of the physiologically active proteins such as the serum proteins, spin down at a rate which is characteristic of molecules which are more compact. These are the *globular* or *corpuscular* proteins, and although some of their

molecules are far from spherical, they do pose special structural problems quite different from those of the fibrous proteins.

The Fibrous Proteins: The structure of the fibrous proteins is fairly clear in its essentials. We have seen that if a single polypeptide chain is written out, taking due account of the valence angles, it appears as a zig-zag chain of carbons and nitrogens. The R-groups of the amino acids then protrude, alternately above and below the plane of the main chain which lies, as represented, in the plane of the paper.



According to all available physical and chemical evidence, this is a valid picture of the actual arrangement of the atoms in such a simple fibrous protein as *fibroin* from silk. This molecule is estimated to be 100 to 200 times as long as it is thick. X-ray diffraction pictures make it possible to measure the "identity period," of such a compound, that is, the distance from one group to an exactly similar one on the same side of the chain. These distances indicate that the bond distances are the normal ones for carbon to carbon, and carbon to nitrogen links in such a chain as is indicated in the diagram. In fibroin over 60 per cent of the R-groups consist of the hydrogen of glycine or the methyl group of alanine. If these are arranged alternately the hydrogens will all be on one side of the polypeptide backbone and the methyl groups on the other. The actual silk fiber consists of several such polypeptide chains laid parallel to each other and twisted about the long axis to form a micelle. The distance between chains in the micelle is small, as would be expected in a compound in which most of the projecting R-groups are very small.

The protein from wool is somewhat more complicated. Its amino acid content is more varied, and like wool itself it can be stretched in water or steam to twice its original length. X-ray data indicate that wool keratin, stretched to its limit, has a structure very like that outlined for fibroin except that its amino acid side chains are much more varied, including those of arginine, cysteine, two dicarboxylic acids, and two of the leucines. Keratin in this stretched form is known as β -keratin.

In the unstretched or α -keratin the polypeptide chain is believed to be coiled or folded in some way. This is indicated by the fact that the identity period in the α -form is just one-half the same period in the β -keratin. But although it is generally agreed that most native protein fibers must

have a folded structure of some sort, the exact form of the folding is still a matter of very active discussion.

Beginning about 1940, W. T. Astbury⁷ made outstanding contributions to that discussion. His x-ray diffraction data for proteins are an important part of the experimental foundation on which speculations about protein structure are being built. It was Astbury who first realized the existence of the α - and β -forms of wool keratin, and he has from time to time suggested various possible folded structures for the α -form.

To be acceptable today a structure for α -keratin must not only be compatible with x-ray data, but it must take into account the facts derived from infrared absorption spectra, from measurement of bond distances and angles, and from calculations of bond energies as well as the more obvious limitations imposed by the actual size and configuration of the R-groups. This problem has been attacked on both sides of the Atlantic and has already given rise to a voluminous literature. At present there are two suggested configurations which seem most nearly compatible with the known facts. One of these, originally proposed by Ambrose and Hanby in England in 1949, postulates a polypeptide chain folded into a series of seven-membered rings. The other, put forward by Pauling and his colleagues at the California Institute of Technology, pictures a coiled polypeptide chain.

The ring folding is illustrated in Figure 5.10. The heavy lines indicate the bonds which make up the polypeptide backbone. The folds in the chain are stabilized by the hydrogen bonds which form between the carbonyl group of one acid and the imino group of the next acid but one in the chain. Since each acid is thus linked to the second acid beyond it and since the ring is a seven-membered ring, this type of fold is known as the 2_7 fold. It is also sometimes spoken of as the α_{II} fold.

In a natural fiber there are several such folded chains, cross-linked to each other through the projecting side chains. In wool, for example, the formation of disulfide links between cysteine residues is believed to contribute largely to the strength of the fiber. Other groups also lend themselves to the formation of cross linkages. A free carboxyl group of a dibasic acid may be esterified by the hydroxyl group of serine or it may form a peptide bond with a free amino group. All such bonds lend rigidity and strength to a protein fiber.

The side chains not only determine the types of cross links which can be formed in a fiber but also how closely the main chains can be packed in a micelle. X-ray data indicate that the distance between chains in fibroin, with its high percentage of simple acids, is only 4.6 to 6 Ångström

⁷ William Thomas Astbury began his professional career at University College, London, but has been at Leeds University since 1928. He has been chiefly interested in the structure of fibers and crystals particularly as they can be studied in x-ray diffraction pictures.

units, while the more complex acids in wool protein hold the main polypeptide backbones at 9.5 Ångstrom units from each other.

A second type of structure which is compatible with the experimental data on proteins was proposed by Pauling⁸ and his colleagues in 1951. In this formulation the polypeptide chain is shortened, not by folding but by coiling to form a helix. Helical forms had been suggested before but always with an integral number of amino acid residues for each turn of the helix. Pauling's computations showed however that in order to fit the experimental data and to form the maximum number of hydrogen bonds

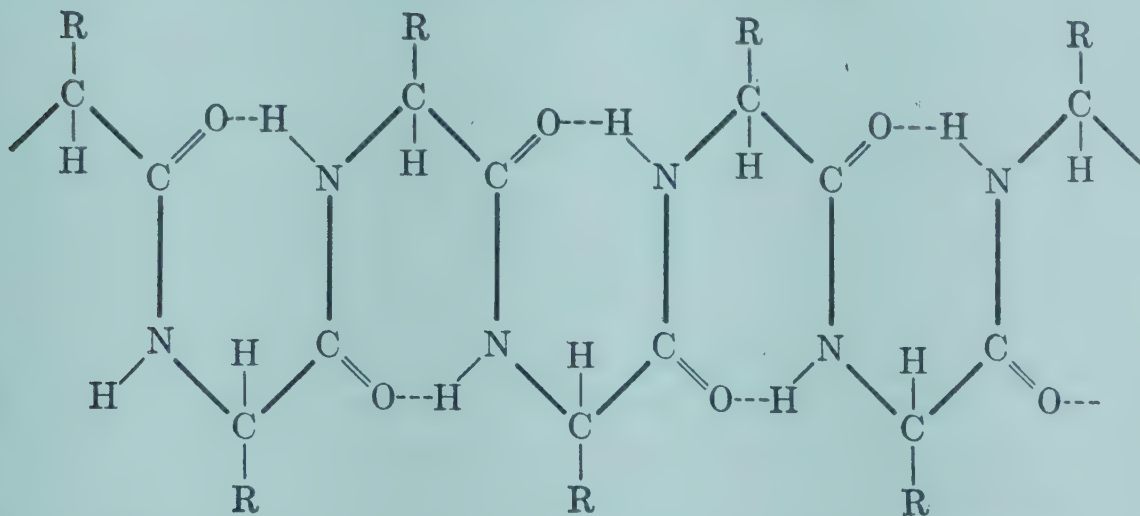


Figure 5.10. The 27 fold. The heavy lines indicate the bonds of the polypeptide backbone. The dotted lines show where hydrogen bridges give stability to the fold. (From C. Robinson and E. J. Ambrose, *Trans. of the Faraday Soc.*, 48:854, 1952.)

such a helical fold must use a nonintegral number of residues per turn. He showed that one with 3.7 residues was compatible with the known facts and that another with 5.1 residues would also be expected to be stable. In these forms the shortened chain is stabilized by formation of hydrogen bonds which unite every carbonyl group in the chain to an imino group of another acid residue. The 3.7 residue helix is an elliptical cylinder with major and minor dimensions of about 13Å and 10Å. Within this cylinder the side chains are laced together through disulfide and other cross bonds, while other side chains jut out from the cylinder to form subsidiary links with other helical polypeptides in the micelle.

The present situation then is that the subject of the structure of α -proteins is in a state of flux. It may be that the polypeptide chain is folded, or it may be coiled. It may be neither or conceivably it may be a combination of both, either simultaneously or in sequence. The last chapters of this complicated story are still to be written, and are appearing in serial form in current journals.

⁸ Linus Pauling is Professor of Physics at the California Institute of Technology. He has made fundamental contributions to the studies of the size and structure of crystals, molecules and ions and to the application of quantum mechanics to chemistry.

The Corpuscular Proteins: Their behavior in the ultracentrifuge indicates that many of the physiologically active proteins such as the serum proteins and hemoglobin are quite different in shape from those just discussed. These are known as the globular or corpuscular proteins though many of them are far from being truly spherical. X-ray studies of hemoglobin show that this molecule is cylindrical in shape, having a radius of 28.5\AA and a height of 34\AA . Table 5-II gives approximate dimensions for four serum globulins.

TABLE 5-II. DIMENSIONS OF GLOBULINS

Length (\AA)	Diameter (\AA)
150	38
300	50
185	185
235	44

Three of the four are clearly unsymmetrical but all are far more compact than the molecules of fibrous proteins. This makes it much more difficult to picture them as long polypeptide chains, yet the evidence all indicates that the acids in the corpuscular proteins are linked exactly as they are in fibroin, through a succession of peptide bonds. The corpuscular shape must arise therefore from some kinking or folding of the chain. There is at present no agreement on the type of folding involved.

Some years ago Dorothy Wrinch offered her "cyclol" theory as a possible explanation of the structure of globular proteins. If the backbone of a hexapeptide were folded as indicated by the heavy lines in Figure 5.11(A), and the terminal carboxyl were to form a sixth peptide bond with the terminal amino group, the molecule would become a large, buckled ring compound. In this form each carbonyl group lies close to the imino group of another peptide bond, and by a simple keto-enol shift could complete hexagonal cells as indicated in (B) by the dotted lines. Wrinch has worked out similar patterns for enormously larger polypeptides and even for such a protein as insulin with a molecular weight of 12,000. In these structures the hexagons are disposed in a plane, and the R-groups jut out above and below this plane. In order to transform such a flat structure into a globular molecule one need only think of the flat sheet's being folded to make the surfaces of a solid figure, as one can fold a triangular sheet of paper to form a tetrahedron.

The cyclol theory is no longer seriously considered but it served one important purpose nevertheless. It opened up the whole question of the actual disposition of the atoms of a protein molecule in space, and by its controversial nature set many people thinking about the problems involved.

The available information indicates that the native globular proteins consist of polypeptides in the α - or contracted form folded backwards and forwards upon themselves. One such possible structure is represented in Figure 5.12. The solid ribbon-like part of the diagram represents the

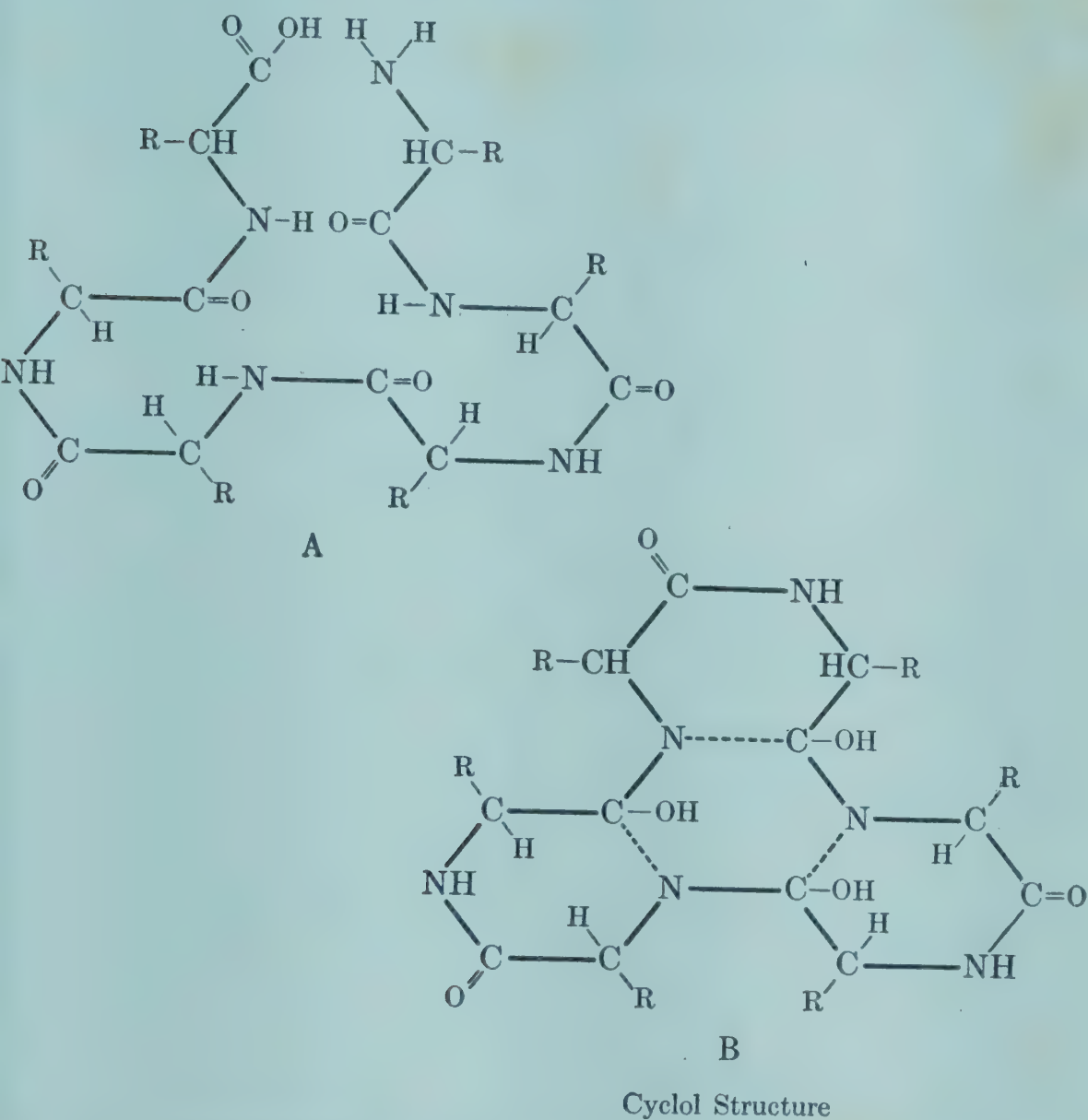


Figure 5.11. Diagrams showing how a polypeptide made up of six amino acid residues could be folded to give a compact structure which would be stabilized by enolization of alternate carbonyl groups, with a resultant formation of internal bonds. (After Wrinch.)

outer dimensions of the polypeptide backbone folded in the 2_7 pattern. This would have a cross section approximately 12\AA by 5.5\AA . The straight lines represent the direction of the first carbon-to-carbon bond of the side chains which jut out in the usual zig-zag "straight chain" form above and below the plane of the seven-membered rings. If the contracted form of α -proteins is the helical one suggested by Pauling, the globular molecule could still be formed by just such a folding back and forth of the long

cylindrical helix. Pauling has suggested that the globular proteins consist of several layers of this general sort piled up "like a stack of pancakes" and held together by interchain links between side chains. Some proteins, of which globin is one, give evidence of having just such a laminated structure. It should be noted that this formulation ascribes to a protein molecule a kind of honeycomb structure, with a good deal of open space between the links which hold the folded backbones together.

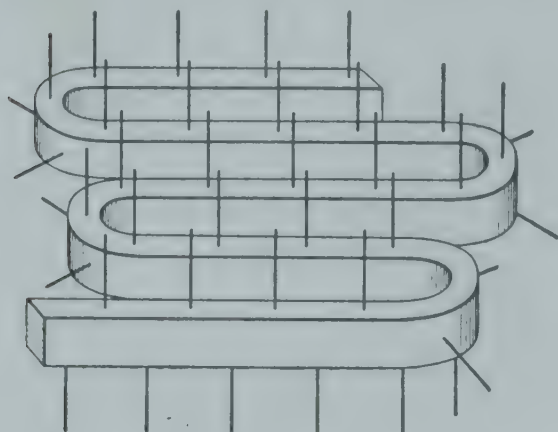


Figure 5.12. Secondary folding in one plane. The solid, ribbon-like part of the figure represents the contracted polypeptide of the 27 fold. This is then folded back and forth to give a thin flat disk which may be one of several such structures making up a corpuscular protein. The short lines represent the direction of the first C—C bond of the side chains (R groups). (From C. Robinson and E. J. Ambrose, *Trans. of the Faraday Soc.*, 48:854, 1952.)

The Order of the Acids. Another question which arises in relation to protein structure concerns the order in which the amino acids are arranged in the polypeptides. It has been indicated that proteins differ both in their amino acid composition and in the molecular ratios in which the acids are present. In one sample of lactoglobulin, for example, there were found 20 serine residues, 36 from aspartic acid, and 50 from leucine. Cysteine, on the other hand, appeared only 4 times, glycine 8, and histidine 4, with several others present in intermediate ratios. Bergmann and Niemann suggested that the acids in such a chain are arranged to give a repeating pattern so that all acids are spaced equidis-

tantly along the chain. With many proteins the actual analytical results can be interpreted very well in terms of such a theory. With a few others the theory predicts a chain in which more than one acid is assigned a single position. Much more experimental work must be done before any definite conclusions can be reached on this complicated subject. It does however seem highly likely that the protein molecule is built on some regular plan which would include a definite order among the amino acid residues.

An interesting new method of determining the arrangement of acids in a protein is the one originated by Sanger⁹ in his study of the relatively small protein, insulin. An intact protein is treated with 2,4-dinitrofluorobenzene (see p. 161) which reacts with any free amino groups. These will be found in those amino acids which are at the amino ends of peptide chains, as well, of course, as the ϵ -amino group of any lysine which happens to be present. Since the link between the aromatic ring and the nitrogen is stable, it is then possible to hydrolyze the dinitrophenyl (DNP) protein to the amino

⁹ Frederick Sanger (1918-) is a member of the external scientific staff of the Medical Research Council. He is working at present at the Biochemical Laboratory in Cambridge, England.

acid stage without removing the substituent groups. This gives rise to free amino acids except that those which had free amino groups in the protein now appear as the yellow DNP-derivatives. The acids can be separated by chromatography and the labeled ones identified by comparison with known DNP-amino acids. By combining this technique with partial hydrolyses of varying degrees of completeness, Sanger has shown that insulin consists of four peptide chains held together by $-S-S-$ bridges from a cysteine residue in one chain to a similar residue in a neighboring chain. Two of the chains have glycine at the amino ends and two have phenylalanine. The complete sequence of amino acids has now been worked out for both types of chain, the phenylalanyl chain consisting of about twenty-nine residues and the glycylic chain of about twenty. For this particular protein there is no obvious periodicity of the amino acids in the chains.

Denatured Proteins. When the properties of a protein have been slightly changed by some chemical or physical agent, but its molecular weight remains unaltered, the protein is said to be *denatured*. Denaturation may be brought about in many different ways such as exposure to heat or x-rays, by brief contact with very dilute acid or alkali, by treatment with urea or guanidine solutions, or even with some proteins by vigorous shaking. The most obvious evidence of denaturation is a decrease in solubility, such as is seen when egg albumin coagulates on being heated. Sometimes the denatured protein, though giving no such clear evidence of change, yet loses its biological activity. Denatured virus is no longer an infective agent; denatured antigen cannot stimulate antibody formation. It seems likely that denaturation is the first step in protein hydrolysis, for denatured proteins show an increased susceptibility to enzymic hydrolysis. In addition to these biological evidences of change there is frequently chemical evidence also. Most native proteins give no test for free sulfhydryl or phenolic groups. When these proteins are denatured, the presence of such free groups is readily detectable by color tests. On the basis of these facts, denaturation has been defined as "any non-proteolytic modification of the unique structure of a native protein giving rise to definite changes in chemical, physical or biological properties."

In the light of what has been said about the structure of native proteins, it is possible to draw a reasonable picture of what may happen when a protein is denatured. In the native protein there is a long polypeptide chain, coiled or folded upon itself in a very definite pattern. Some of the side chains or R-groups will be folded in, forming cross-links with other interior side chains. These will build up inside the molecule a kind of lattice work in which the gratings consist of such linkages as result from formation of esters, of disulfide bonds, and of supplementary peptide linkages. It is not hard to see why any tampering with the elaborate pattern so set up would change the properties of the molecules.

According to present theories, denaturation consists of a destruction or

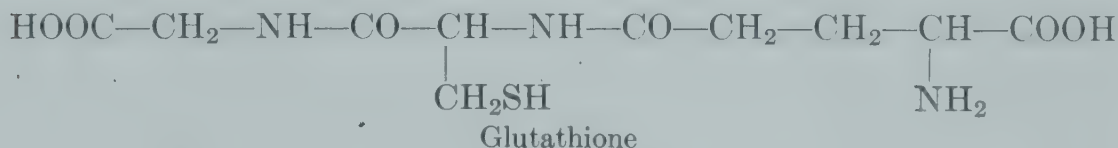
partial destruction of that unique pattern. This may range from the breaking of a few minor cross-links to such disorganization that the polypeptide chain becomes completely extended, or at any rate, folded only in a haphazard fashion. In the process certain groups may actually be freed. Sulfhydryl groups would appear if disulfide bonds were opened. Free carboxyl, amino, and hydroxyl groups would appear wherever amide and ester cross-links were hydrolyzed. But not all groups which become manifest as a result of denaturation need actually have been "formed" by ruptured linkages. A free sulfhydryl group, for example, might be so buried within a molecule, or so hemmed in by large adjacent groups, that no nitroprusside (see p. 205) could reach it. After denaturation the molecule is disoriented and its chains at least partially extended. In this condition the sulfhydryl groups might be readily available for reaction with nitroprusside and so would seem to have been newly freed.

Nonprotein Compounds of Nitrogen

In addition to the proteins, there are in living tissue other nitrogenous compounds which are of biological importance. The place of some of these substances in the chemistry of cell metabolism is known; the importance of others is inferred from their wide distribution in living material though their exact function remains to be discovered.

GLUTATHIONE

Although isolated native proteins give no test for free sulfhydryl groups, living tissues quite uniformly respond to the nitroprusside test. Search for the cause of the positive test revealed the presence in nearly all living cells of a tripeptide named "glutathione" by Hopkins¹⁰ who first isolated it. Glutathione consists of residues of glycine, cysteine, and glutamic acid condensed to form a tripeptide.



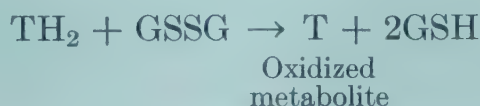
Since the important reactions of the molecule center about the sulfhydryl group, the abbreviation GSH is frequently used, and its ready reversible oxidation is then indicated:

¹⁰ Sir Frederick Gowland Hopkins (1861-1947) was an outstanding member of the small group of scientists who at about the turn of the century gave biochemistry the standing of an independent discipline. His own brilliant contributions to the science were a result of a rare combination of meticulous chemical technique and insight, with a biologist's feeling for the cell as a complex living organism. As Head for many years of the Sir William Dunn Institute of Biochemistry at Cambridge he attracted students from all over the world and fostered research on a wide variety of biochemical problems. He was awarded every scientific honor which England has in her gift, and was a co-recipient of the Nobel Prize in Medicine in 1929.



Since its discovery in 1921 many attempts have been made to assign a significant biological function to this compound. It has proved to be an essential participant in one or two enzymic reactions, but so far it has not been shown to be an obligatory component of any of the major metabolic systems. On the other hand, there are many enzymes which depend for their activity on the presence of free sulfhydryl groups in the enzyme molecule. Since such groups are readily oxidized, and the resulting enzyme is entirely inactive it may well be that one function of glutathione is to keep these enzymes in reduced form.

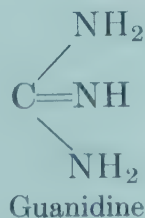
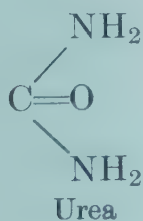
Glutathione has also a theoretical importance from the circumstance that it was through a consideration of its reversible oxidation-reduction that Hopkins in 1926 was led to postulate what he described as a "ferrying" action. Suppose that TH_2 represents some metabolite in the cell which is to react with oxygen and form water. For this substance oxidized glutathione could act as a ferry by accepting two hydrogen atoms to form GSH and then passing them on to molecular oxygen. This would reconstitute the oxidized glutathione molecule which could then pick up another pair of hydrogens from another molecule of the metabolite. The net result would be oxidation of the metabolite and the formation of water. Actually no such specific function for glutathione has ever been discovered. On



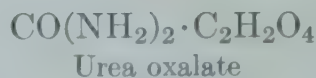
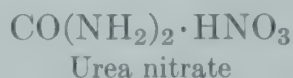
the other hand, this suggested mechanism for the course of oxidative reactions proved enormously fruitful in work with cell oxidizing systems. Cyclic changes of just the type suggested have proved to be very common in the chemistry of living tissues.

UREA AND GUANIDINE

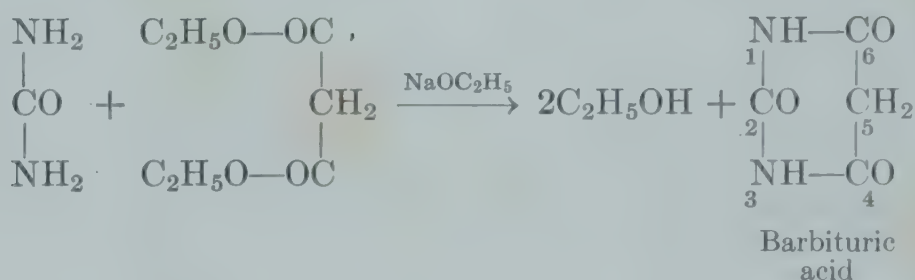
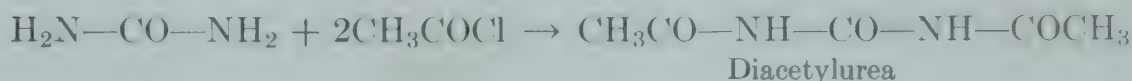
In man and in some other species, the end product of nitrogen metabolism is urea, the diamide of carbonic acid. Another important nitrogen compound is the much more strongly basic substance guanidine. The relation between the two compounds is evident from their formulas. It is the δ -guanido group in arginine which makes it a basic amino acid.



Urea forms molecular compounds with acids in the cold. The characteristic forms of the saltlike crystals of these compounds are sometimes used to identify urea in biological fluids.



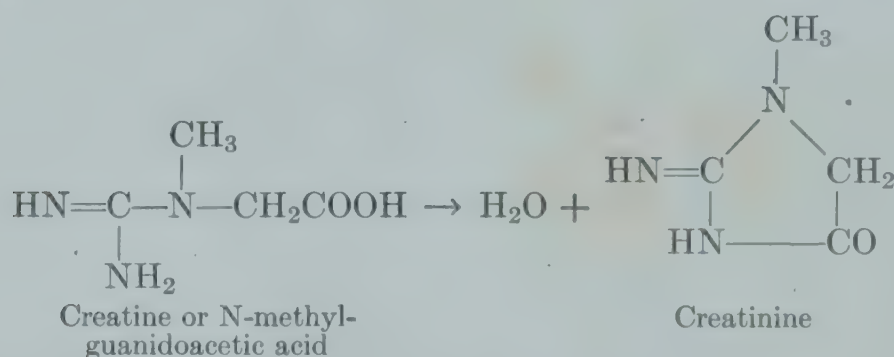
If, however, urea is treated with an acid chloride instead of a free acid, a condensation takes place, yielding a compound known as a *ureid*. The product of the reaction with acetyl chloride is a straight chain compound, but with the dibasic acids the important cyclic ureids are formed. For example, diethyl malonate condenses with urea in the presence of sodium



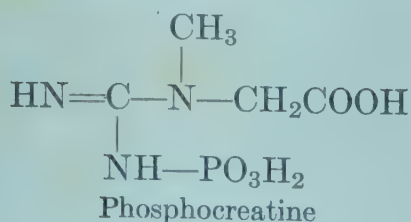
ethoxide to form the six-membered ring compound, malonyl urea or barbituric acid. Derivatives of barbituric acid, in which one or both hydrogens on carbon 5 are substituted, are important soporifics. Veronal, for example, is diethyl barbituric acid. This particular arrangement of carbons and nitrogens in a large ring is found in a number of compounds of great biological importance. Two compounds of the vitamin B-complex, B₁ and B₂, each contain this ring as do the bases which make up part of the nucleic acid molecule.

CREATINE AND CREATININE

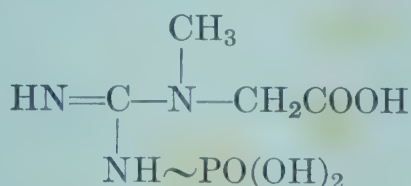
Related to guanidine is the basic substance, creatine, and its anhydride, creatinine.



The phosphoric acid derivative of creatine, sometimes called phosphagen, plays an important part in muscle metabolism.



The linkage between phosphorus and nitrogen in phosphocreatine is an example of the type of bond which is spoken of as an "energy rich" bond. Hydrolysis of this bond yields over 10,700 cal. per mole. This is to be compared with about 2500 cal. set free by splitting a typical phosphate ester linkage, such as the one in glucose-6-phosphate. The energy rich bond is differentiated from others involving less energy by use of a wavy



line. One of the prime functions of oxidation in the muscles is provision of the energy necessary for formation of such energy rich phosphate bonds.

NUCLEIC ACIDS

The nonprotein nitrogen compounds so far considered are all simple substances, the formulas of which are known. The nucleic acids, on the other hand, are macro- or giant molecules whose structures are still to be worked out.

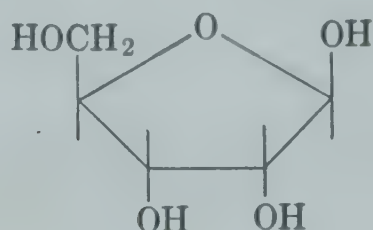
Preparation of the Nucleic Acids. All living cells contain conjugated proteins which were originally called "nucleoproteins" because they were believed to be the characteristic proteins of cell nuclei. They are especially abundant in spermatozoa and in glandular tissue such as thymus, but are found in the cytoplasm as well as the nuclei of all sorts of cells, including bacteria and yeasts. Nucleoproteins from different sources have certain properties in common, but also differ markedly from each other. That they are essentially different substances is indicated by the fact that there is no general method of extraction of nucleoprotein. Each tissue presents its own special problem, enormously complicated by the fact that very little is known about the stability of the substance to be extracted. Undoubtedly, many of the earlier preparations consisted of molecules more or less degraded in the course of their extraction and purification. Of recent preparations, probably the plant viruses which are extracted at very low temperatures have suffered least damage.

When any nucleoprotein is hydrolyzed very gently it splits into a protein part and a prosthetic group which is called nucleic acid.

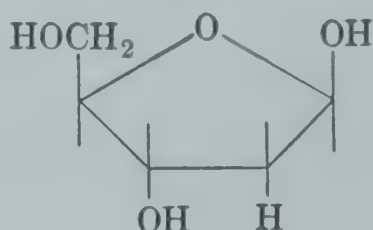


This may be brought about by mild alkaline hydrolysis at room temperature, or by denaturation of the protein, or by peptic digestion. The alkali salts of the nucleic acids are water soluble. The acids themselves are precipitated when the solution of their salts is acidified.

Hydrolysis of Nucleic Acids. So far two types of nucleic acid have been distinguished, but it should be emphasized that this does not mean that all the acids of a single type are identical. When any nucleic acid is hydrolyzed completely, by acids or by enzymes, the products are phosphoric acid, a sugar, and a mixture of basic substances. From some acids the sugar obtained is a pentose; from others a desoxypentose is set free. All the sugars which have been characterized have proved to be either ribose or desoxyribose, both in the furanose form. Since, however, there are many nucleic acids in which the sugar has not been unequivocally



β -D-Ribofuranose



2-Desoxy-D-ribofuranose

identified it is better for the present to classify the known nucleic acids as either pentose or desoxypentose types. Of the pentose-containing nucleic acids that from yeast has been most thoroughly studied. Thymus nucleic acid is the best known of the desoxypentose compounds. Complete hydrolysis of these two acids yields the products listed.

YEAST NUCLEIC
ACID

Phosphoric acid

D-Ribose

Adenine

Guanine

Cytosine

Uracil

purine bases

pyrimidine bases

THYMUS NUCLEIC
ACID

Phosphoric acid

D-2-Desoxyribose

Adenine

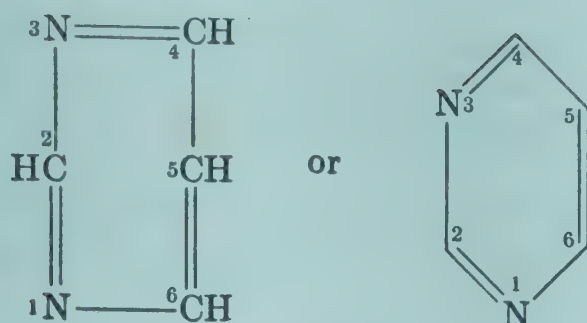
Guanine

Cytosine

Thymine

The mixture of bases obtained by hydrolysis of a nucleic acid has always so far consisted of two purine bases, adenine and guanine, and two pyrimidine bases, cytosine and uracil from the pentose acids and cytosine and thymine from those in which the sugar is a desoxypentose.

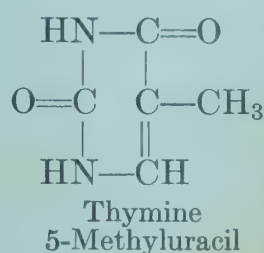
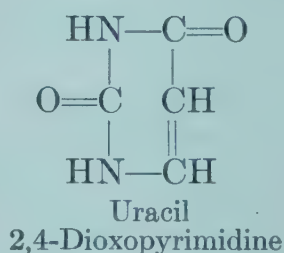
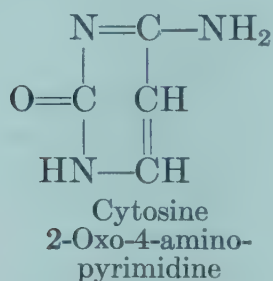
Pyrimidine Bases. It is customary to say that the parent heterocyclic compound of this group of bases is pyrimidine, which it will be noted has the same ring form that is found in barbituric acid. In actual fact several



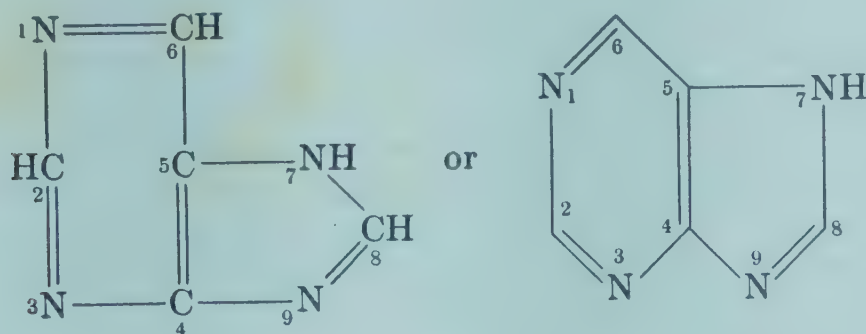
Pyrimidine

of the "derivatives" were synthesized or discovered before it was suggested that they might all be considered to be substitution products of the simple ring compound to which has been given the name pyrimidine.

The relation of those pyrimidine bases which occur in nucleic acid to each other and to pyrimidine itself is indicated in the formulas. It should be noted that when the ring includes a carbonyl carbon the neighboring nitrogen holds a hydrogen, and there is one less double bond in the ring.



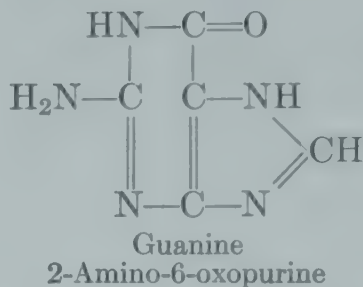
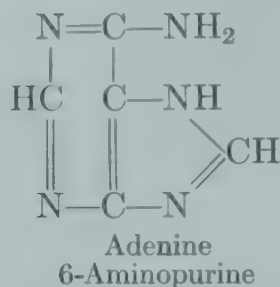
Purine Bases. The first of this group to be isolated was uric acid, separated from urinary calculi and from mammalian urine by Scheele in 1776. When it had become clear, toward the end of the nineteenth century, that the same fundamental ring structure was common to uric acid, caffeine, and several other basic substances extracted from living tissue, Emil Fischer suggested that they all be named as derivatives of a hypothetical substance to which he gave the name "purine."



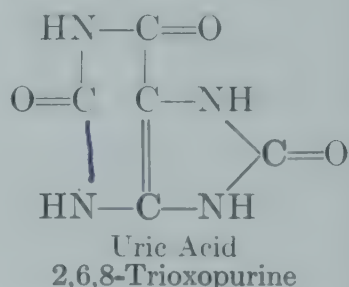
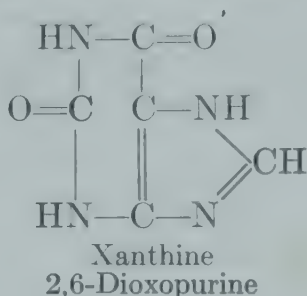
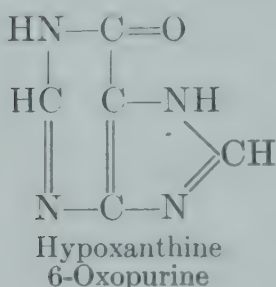
Purine

The purine bases consist of two condensed heterocyclic rings. The six-membered ring is identical with the pyrimidine ring, though no longer

numbered in the same sequence! The five-membered ring may be thought of as resulting from condensation of a reduced urea at carbons 4 and 5. Thus the purines are reduced cyclic diureids. The five-membered ring is

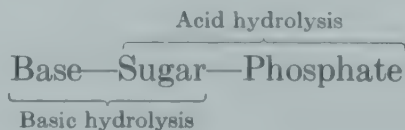


the same one which appears in histidine and is known as an imidazole ring. The formulas given indicate the close structural relationship between the various purine bases which occur in tissue or in body fluids.

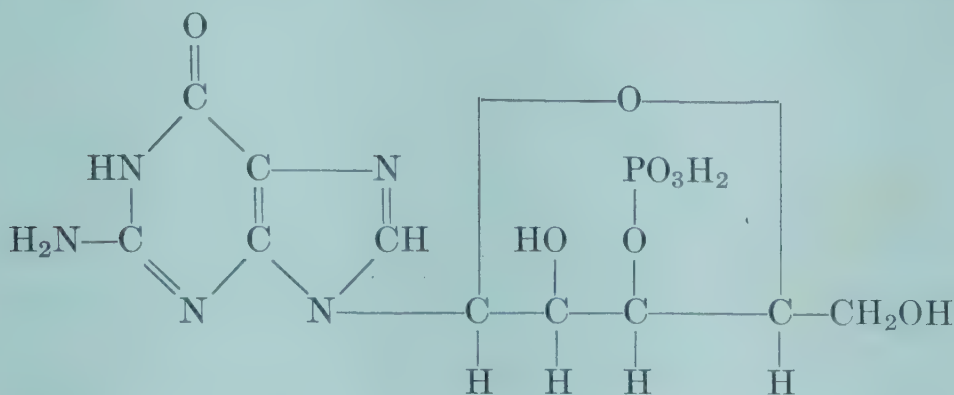


Both the purine and the pyrimidine bases have high extinction coefficients for ultraviolet light of about 2600Å. This fact is used to show the location of nucleoproteins in the cell. When ultraviolet light of the proper wave length is used in the quartz microscope to illuminate a thin slice of tissue, regions of high nucleoprotein concentration appear dark in the photomicrograph of the tissue, while other cell proteins transmit most of this light and appear bright.

Nucleotides. If a nucleic acid is subjected to less drastic conditions than those which lead to complete hydrolysis, various fragments may be identified which throw light on the structure of the acid. Yeast nucleic acid, for example, is split either by a specific pancreatic enzyme or by prolonged, mild alkaline hydrolysis at room temperature to a mixture of four different *mononucleotides*. These are compounds of phosphoric acid, ribose and one of the four constituent bases. If the further degradation of a mononucleotide is carried out in alkaline solution, phosphoric acid is freed, leaving a base-sugar compound known as a *nucleoside*. Very mild acid hydrolysis, on the other hand, splits off the base, leaving the sugar-phosphate link intact. These results indicate that the sugar lies between the phosphoric acid residue and the base.

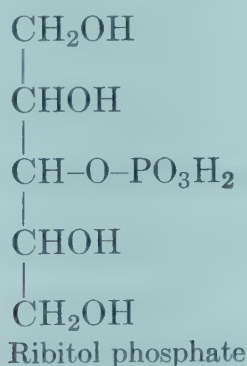


Recent work has defined somewhat more completely the structure of the nucleotides, both from yeast and from thymus nucleic acids. The yeast mononucleotides prove to have the phosphate group attached to carbon 3 of ribose, while the base is linked through nitrogen to carbon 1 of the sugar. The pyrimidine bases are linked at position 1, the purines at position 9. Guanylic acid will serve as an example of a purine ribonucleotide.



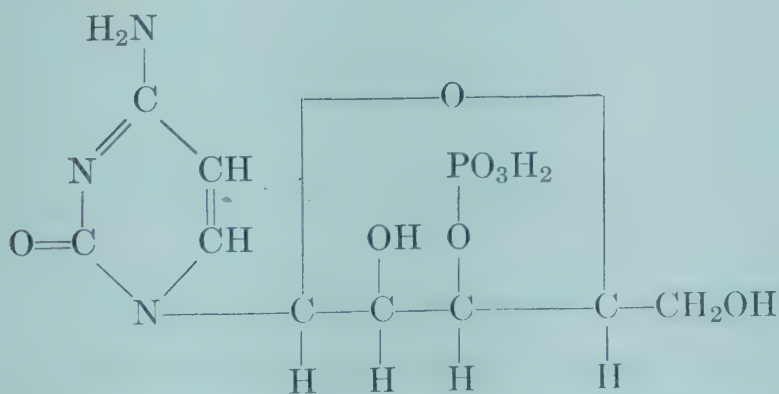
Guanylic acid or 9-Guanine-3-phosphoribofuranoside

The position of phosphoric acid on carbon 3 was proved by reduction of the ribose phosphate which resulted from mild acidic hydrolysis of the nucleotide. The ribitol phosphate which formed was optically inactive, showing that the phosphate must have been esterified at the middle hydroxyl group, thus making the compound perfectly symmetrical.



Ribitol phosphate

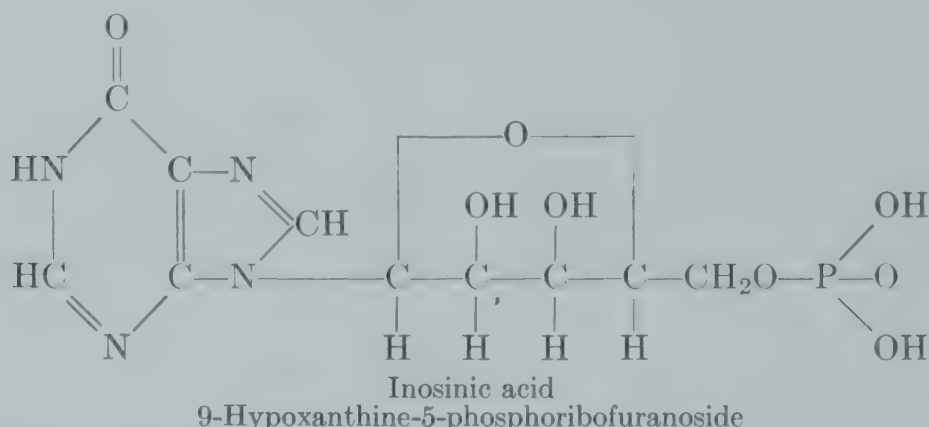
In cytidylic acid the pyrimidine base is cytosine.



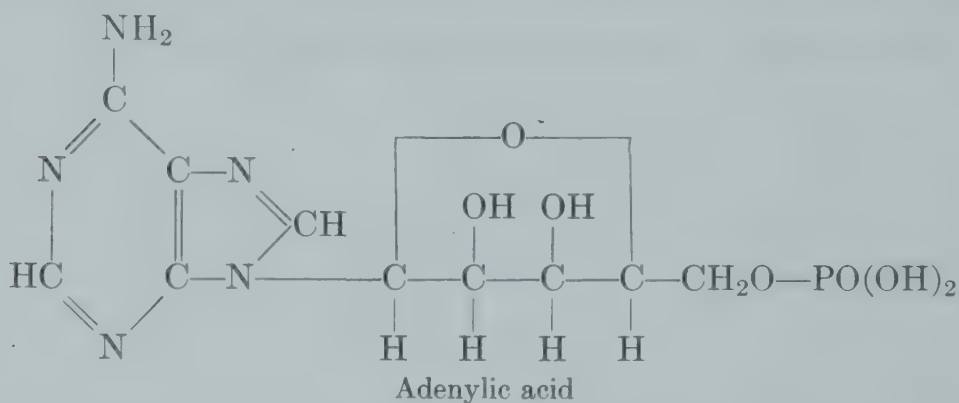
Cytidylic acid or 1-Cytosine-3-phosphoribofuranoside

So far the position of the phosphate group in the nucleotides which contain the desoxysugar has not been determined.

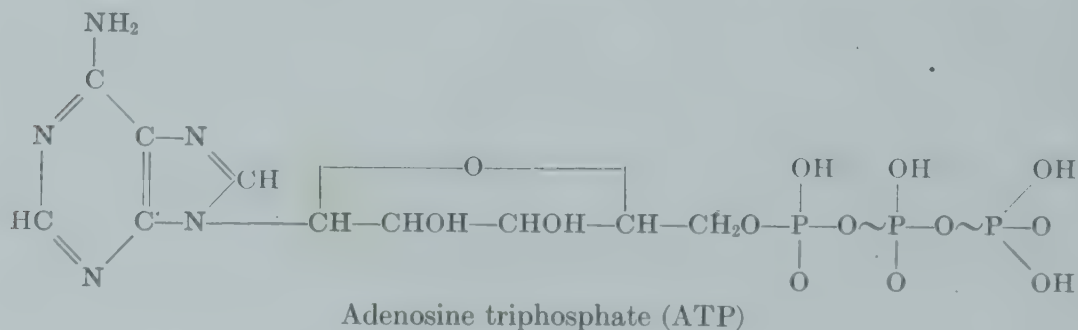
There are several mono- and dinucleotides which occur free in living tissue and play an essential role in tissue metabolism. The first of these to be isolated was inosinic acid which Liebig prepared from beef extract. The base in this compound is hypoxanthine, and phosphoric acid is linked to carbon 5 of the ribose.



Adenylic acid is another mononucleotide which occurs free. It is found in muscle, and plays an important part in muscular contraction.

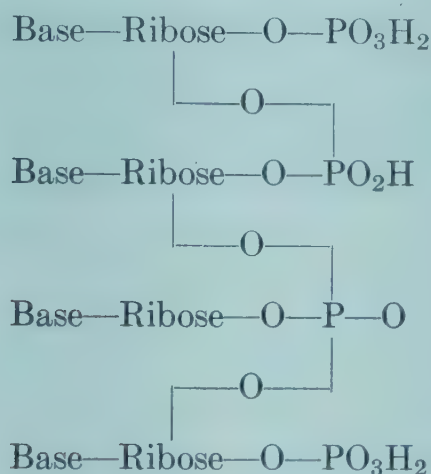


The pyrophosphate derivative of adenylic acid is one of the most important of the compounds which contain energy rich phosphate bonds.



Structure of the Nucleic Acids. Until very recently the nucleic acid molecule was believed to consist of four different mononucleotides, two con-

taining purine bases, and two with pyrimidine bases. The four were supposed to be linked together to form a tetranucleotide. This structure was based upon the following facts: (1) Hydrolysis of nucleic acids seemed to liberate always equimolecular amounts of the four constituent bases, and always in the ratio of two purines to two pyrimidines. (2) A determination of the molecular weight of the acid in 1938 gave a figure which seemed to indicate a molecule of about tetranucleotide size. (3) Titration of the acid indicated four or five free acid hydrogens in the phosphate radicals. This was best explained by a structure in which one mononucleotide was linked to another through a phosphoric ester linkage from the phosphate of one nucleotide to an hydroxyl group of the sugar in another. It had even seemed possible on the basis of their relative rates of hydrolysis to decide which nucleotides were the outer ones and which were the central ones. In accordance with the available evidence, various structures were proposed for the tetranucleotide, some of them open chain compounds and one, at least, a kind of giant ring. Gulland, for example, in the light of his work on electrometric titration of a nucleic acid, proposed the formula below as one of three possible arrangements which would fit the figures:



Within the last few years it has become necessary to discard entirely any such simple formula. Methods now in use for isolation of the nucleic acids are far less drastic than were some of the earlier ones, and it may be for this reason that the acids now appear to have enormously higher molecular weights than those on which the tetranucleotide structure was based. A thymus nucleic acid is reported to have a molecular weight between 500,000 and 1,000,000, indicating a polynucleotide containing 1500 to 3000 mononucleotides. The figures for pentose (yeast) nucleic acid are lower, but still indicate molecules made up of 55–116 mononucleotides.

Nor are the molecular weights the only results in need of correction. Until quite recently the methods for isolation of mononucleotides or of the free bases were time consuming and complicated. Paper partition chromatography and spectrophotometry are now being adapted to the estimation

of small amounts of the bases, and bio-assay offers a delicate test for the presence of small amounts of some of the mononucleotides. On the basis of results with these newer procedures, it is no longer clear that the bases are present in the simple ratio originally postulated. Thymus nucleic acid has recently been shown to include bases in the ratio: 10 cytosine:16 adenine:13 guanine:15 (or 13) thymine.

The situation is then that almost nothing is known about the structure of nucleic acid except that it consists of mononucleotides united to form macromolecules. The linkages between the mononucleotides almost certainly involve the phosphate groups, but whether these are linked to the sugar of a neighboring nucleotide or to the base is not known. One sample of a thymonucleate is described as having "a chain structure . . . with only very infrequent branching," and all the evidence points to asymmetric molecules, many times as long as they are wide. The wide variation in the molecular weight figures for different samples of nucleic acid may indicate the existence of many different nucleic acids of varying complexity. On the other hand, it may simply reflect different amounts of destruction of the original molecules in the course of extraction and purification.

There is a certain tendency apparent in the literature to speak of the nucleic acids as polymerized tetranucleotides. There is no experimental justification for this, and it seems to be simply the superposition upon the older concept, of recent ideas about the size of the macromolecules. Until more is known about them, any definite pictures of units larger than mononucleotides are bound to be illusory.

Importance of the Nucleotide Structure. It has been known for many years that mono- and dinucleotides perform unique functions in various metabolic cycles. Reference has already been made to adenylic acid and its pyrophosphate derivative. There are also found in living tissue a number of compounds having the structure of two nucleotides, united to each other through their phosphate groups. These substances, in which are found other bases than those which occur in nucleic acids, have proved essential to the activity of various oxidizing enzymes. They will be considered in connection with cell oxidations. Meantime, let us glance briefly at two nucleoproteins which exemplify the special importance of these substances.

Viruses: The larger infectious agents such as bacteria and amoebae readily reveal themselves under a microscope, and are held back if a liquid in which they are suspended is passed through an unglazed porcelain filter. But a number of plant and animal diseases are caused by sub-microscopic particles which are not held back by a filter and which are therefore known as *filterable viruses*. In the presence of the living cells of a suitable host, the viruses multiply and spread and cause characteristic manifestations of disease.

From tobacco plants affected with a mosaic disease, Stanley¹¹ isolated and ultimately crystallized the first example of a pure chemical compound having the properties of a virus. This substance proved to be a nucleoprotein of the ribose type. Even after repeated chemical manipulations it is still an active infective agent capable of multiplication. Thus, it 'stands on the threshold between the living and the never-having-lived' (Gulland).



Figure 5.13. Electron micrograph of tobacco mosaic virus, expressed from infected leaves and washed once with distilled water. (From R. C. Williams and R. L. Steere, *Science*, 109:308, 1949.)

In the past ten years the infective agents of many virus diseases have been isolated. All have proved to be nucleoproteins of very high molecular weight and rather low nucleic acid content. In most compounds so far characterized, the sugar has proved to be a pentose. Tobacco mosaic virus is estimated to have a molecular weight of $25-60 \times 10^6$. The other viruses have molecular weights of roughly this order of magnitude.

The molecule of tobacco mosaic virus has been extensively studied by x-ray diffraction and in the electron microscope. It appears to be made up of minute rods about 2800\AA long and 150\AA in diameter. Within the rods is an intricate structure, with transverse and longitudinal spacings which are interpreted to mean that the protein fabric is made up of little cubes, 1\AA on a side. The cubes, consisting of closely folded polypeptide chains, are built in a regular pattern into larger units, all of which are joined in some way to a long fibrous nucleic acid chain of about 800 nucleotides. The shape of the rods is clearly seen in Figure 5.13, which is an electron

¹¹ Dr. Wendell M. Stanley worked for many years at the Rockefeller Institute in New York and is now in charge of Virus Research at the University of California in Berkeley. He is known best for his isolation of virus proteins. In 1946 he shared the Nobel Prize in Chemistry with J. B. Sumner and J. Northrop.

micrograph of the virus as it occurs in the leaf, following a single washing to remove cell debris. Figure 5.14 shows the appearance of a single purified crystal of the nucleoprotein which has been shadowed with gold to make visible the cubical arrangement of the units within the crystal.



Figure 5.14. Electron micrograph of crystalline tobacco mosaic virus. (From R. Markham, K. M. Smith, and R. W. G. Wyckoff, *Nature*, 161:760, 1948.)

Genes and Chromosomes: Within the nucleus of cells are the tiny thread-like chromosomes which go through a characteristic series of changes leading up to cell division. In the course of those changes each chromosome splits lengthwise and one half goes to each of the two daughter cells. Figure 5.15 shows a chromosome as revealed by the electron microscope. The twisted structure may throw light on the mechanism of the well-known longitudinal cleavage.

It has long been accepted that the chromosomes consist largely of nucleoprotein though there is some evidence that the nucleoproteins fit into some kind of continuous framework of simple protein. The discovery that the nucleotide spacing is of the same magnitude as that between amino acid side-chains in a fully extended polypeptide, has led to the belief that the nucleic acid and polypeptide molecules lie lengthwise along

the chromosomes. X-ray data and absorption spectra indicate that in this structure the purine and pyrimidine rings lie in roughly parallel planes, at right angles to the main axis of the nucleic acid itself. Fibrous nucleic-



Figure 5.15. An electron micrograph of a chicken chromosome after five extractions with M NaCl, showing intertwining and crossbanding. (From A. R. T. Denués, *Science*, 113:203, 1951.)

proteins which have been prepared from cell nuclei contained as high as 66 per cent nucleic acid.

In order to account for the orderly way in which an organism develops and in which hereditary characteristics are handed on, geneticists postulate far smaller structures within the chromosomes. These are the genes, thou-

sands of which are believed to lie along the main axis of the chromosomes. Evidence is accumulating that the gene, like the virus, is a nucleoprotein capable of self-duplication. It is suggested that the genes regulate cell activities by controlling protein synthesis in the cell, thus being able to direct both the reduplication of the genes themselves and the formation of essential enzymes. The way in which this is accomplished is far from clear, but surely it is significant that changes in nucleoprotein concentration can be correlated closely with the various stages which succeed one another in the course of cell division. Regions or times of intense protein synthesis are characterized by high nucleoprotein concentration. It is suggested that the nucleoprotein of a gene may act as a sort of model or templet, against which a new molecule is formed, rather as a casting is made. Conceivably, it could give rise in this way to a perfect copy of itself. Or, since the nucleoprotein is an enormous molecule, it could by building from one or more special areas give rise to an enzyme protein of a different structure which would then direct the next step in the chemistry of the cell.

All of this is still largely in the realm of speculation, but it serves to indicate the way in which biochemical genetics is developing. For further progress in this field the most elegant techniques of genetics, physics, and chemistry will be needed.

Reactions of Analytical Significance

Certain of the reactions which amino acids undergo lend themselves to the determination of the total concentration of free amino acid in a mixture. These are especially important in following the course of protein hydrolysis, and in determining when it has reached completion.

GENERAL AMINO ACID REACTIONS

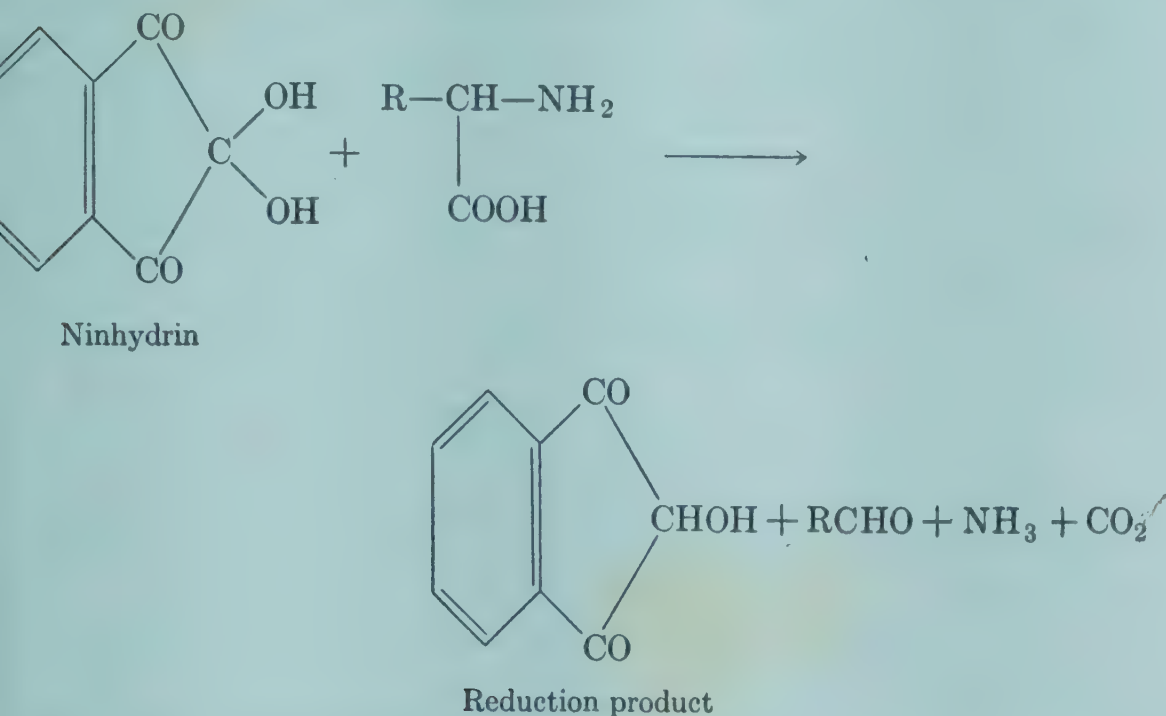
Reference has already been made to two analytical reactions which are common to nearly all amino acids.

By the *Van Slyke* method (see p. 160), using nitrous acid, the total amount of free amino nitrogen may be determined, with corrections for three amino acids. The nitrogen of proline is not evolved in this method, nor is the guanidino nitrogen of arginine. Glycine also behaves anomalously, and this must be allowed for in any mixture known to contain glycine. This method lends itself readily to following the course of protein hydrolysis. A small aliquot sample of a protein solution will give evidence of very little free amino nitrogen. Samples removed from time to time while hydrolysis is proceeding will show gradually increasing numbers of free amino groups until finally all the peptide bonds have been ruptured.

Sørensen's use of formaldehyde (see p. 160) makes possible the estimation of the total free carboxyl groups in a hydrolytic mixture. Formol titration of a freshly made protein solution indicates that there are present

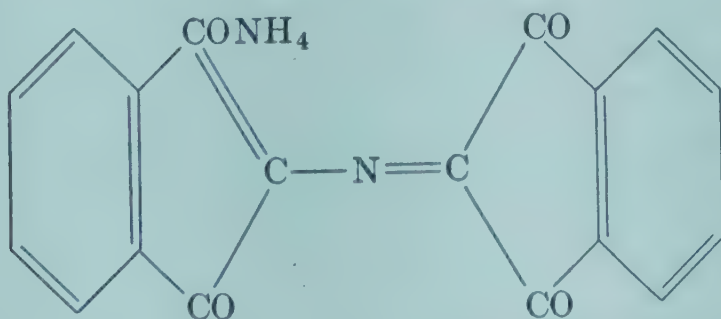
relatively few free carboxyl groups. As hydrolysis of the protein progresses this number increases until all the amino acids have been set free.

The *ninhydrin* reaction depends upon the oxidative deamination of amino acids when they are warmed with triketohydrindene hydrate (ninhydrin).



This reaction has been adapted by Van Slyke, who is fond of gasometric methods, to estimation of the total free amino acids by measurement of the volume of carbon dioxide evolved. If the pH and the temperature are properly controlled, the reaction is specific for free amino acids, including proline and oxyproline.

When the ninhydrin reaction takes place at a pH above 4.7 one molecule of unchanged ninhydrin condenses with the reduction product and ammonia to yield a blue substance believed to be:



In this reaction also, proline and hydroxy proline react, yielding colored condensation products though not a true blue. Because of the introduction of this second color, quantitative estimation of total amino acids by this colorimetric method has not yet been successful. However, when the acids

of a protein hydrolysate have been separated from each other by one of the chromatographic methods (p. 176), it is possible to use the ninhydrin color to determine approximately the amount of the individual acids present.

Pope and Stevens have elaborated a titrimetric method for estimation of free amino acids. It is based on the reaction between α -amino acids and insoluble cupric phosphate to form soluble complex cupric salts (see p. 159). This solution is filtered from excess of the insoluble cupric phosphate reagent, and the amount of cupric ion in solution is determined by iodometric titration. The reaction used is approximately the reverse of the one used in the Shaffer-Hartmann estimation of sugars. The Shaffer-Hartmann method estimates the amount of *cuprous* ion in a solution by adding a carefully measured excess of iodine in the presence of potassium iodide and titrating the residual iodine after the following reaction has taken place:



In the amino acid determinations, the amount of *cupric* ion in solution is estimated by using so high a concentration of iodide ion that the cupric ion is reduced:



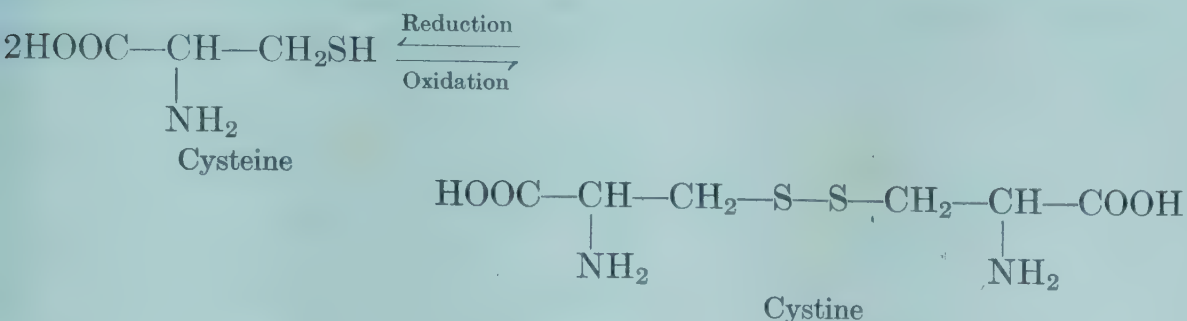
Titration with sodium thiosulfate measures the amount of iodine set free, which is in turn a measure of the amount of cupric ion which formed a salt with the amino acids. This method has two advantages over the earlier Van Slyke nitrous acid method for total free amino acids. It estimates proline and oxyproline, and it is not sensitive to the presence of free ammonia.

REACTIONS OF INDIVIDUAL AMINO ACIDS

Some of the amino acids which have distinctive functional groups take part in specific reactions, often leading to formation of a colored product. Some such reactions have been adapted to testing for the presence of a particular amino acid in combination in the native proteins. These are referred to under "Color Reactions of Proteins" (pp. 205-207).

Other tests can be applied only if the amino acids are free. A number of these have been adapted for qualitative or quantitative estimation of a particular acid in a mixture. It should be noted that most of these tests are not specific for the amino acid but only for a chemical group. For example, a test for "tyrosine" is actually only a test for the presence of a phenolic hydroxyl group, and it is a valid test for tyrosine only if the presence of other phenolic compounds has been ruled out. In a mixture known to consist of natural amino acids, the only phenolic hydroxyl group is that of tyrosine.

Nitroprusside Test. Sodium nitroprusside reacts with a free sulfhydryl group ($-\text{SH}$) in the presence of ammonia to give a purple-red color. Since cystine is easily reduced to cysteine, the test may be used before reduction to indicate the presence of cysteine, and after reduction to determine whether the amount of free sulfhydryl group has increased. If it has, the presence of cysteine is indicated.



The reaction of the nitroprusside is believed to be:



Sullivan's Test. The quantitative estimation of cystine begins with reduction of this acid to cysteine with sodium sulfite. The reagent is sodium- β -naphthoquinone-4-sulfonate, used in presence of alkali. Other amino acids give a similar color but it is only cystine which gives a color that persists after treatment with sulfite. Like many of the colorimetric methods, the validity of this one rests not on strict specificity, but on proof that, of the molecules likely to be present in a protein hydrolysate, only cystine yields the color under the specified conditions.

Folin and Looney's Test. This test for tyrosine makes use of one of the numerous solutions of complex phospho- acids. This particular reagent consists of a mixture of phosphotungstic and phosphomolybdic acids. These acids, singly or together, are reduced by a number of different substances to give a clear blue color of unknown composition. In the "phenol reagent" used for detection of tyrosine, the solution is adjusted as to acidity and reagent concentration so that the appearance of the familiar blue color indicates the presence of a phenolic hydroxyl group.

Sakaguchi's Test. The color test for arginine rests upon a reaction of the reagent with the guanido group of the amino acid. Arginine treated with α -naphthol and sodium hypobromite forms a bright red colored substance which can be used for quantitative estimation of the amino acid.

PROTEIN ANALYSIS

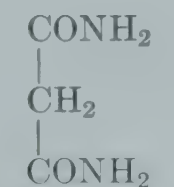
Color Reactions of Proteins. There are a few classical color tests which serve to indicate the presence in proteins of certain linkages or functional groups. The exact composition of the colored product is often not known.

The Biuret Test. This is the most general of the color reactions and is

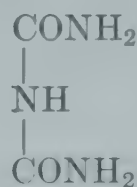
given not only by the proteins but by many of their hydrolytic products, and indeed by many quite simple molecules. If a drop of very dilute cupric sulfate solution is added to a protein solution containing excess of potassium hydroxide, a characteristic violet or rose-violet color develops. The simplest molecule which gives a positive test is one containing two peptide linkages, either directly attached to each other, as in oxamide, or separated by but one carbon or nitrogen atom, as in malonamide or in biuret itself. This last is formed by loss of ammonia when urea is heated to a high temperature.



Oxamide

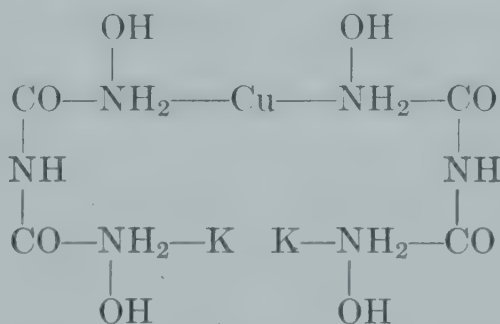


Malonamide



Biuret

The colored substance is a complex, reminiscent of those copper-amino acid complexes formed by free amino acids. Its structure is believed to be:



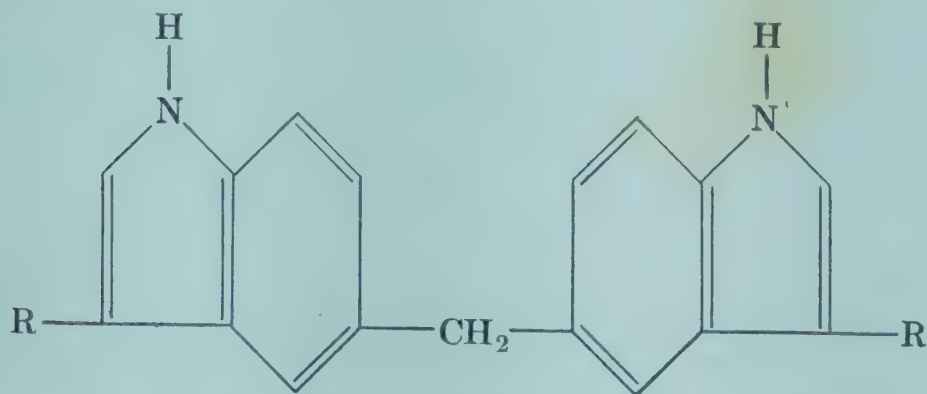
The importance of the test lies chiefly in the evidence it offers for the existence of peptide linkages in proteins.

Millon's Test. Millon's reagent consists of a mixture of mercuric nitrate and mercuric nitrite in excess of the two acids. It cannot be used with alkaline solutions nor in the presence of a high concentration of inorganic salts.

Most proteins when warmed with Millon's reagent develop a brick-red color. A similar though deeper color appears when any compound containing a phenolic hydroxyl group is tested with the reagent. Since the only naturally occurring amino acid which contains this group is tyrosine, the development of the typical color establishes the presence of tyrosine in a protein.

Hopkins-Cole Test. Several different names have been associated from time to time with the color reaction which indicates the presence of tryptophan in a protein. In 1874 *Adamkiewicz* noted that most proteins develop a violet color if treated with glacial acetic acid and concentrated sulfuric acid. A search for the substance responsible for this color led *Hopkins and Cole* to the first isolation of tryptophan in 1901. They proved at this time

that the active substance in the Adamkiewicz reagent was glyoxylic acid ($\text{CHO} \cdot \text{COOH}$), present as an impurity in most samples of acetic acid. The Hopkins-Cole reagent consists accordingly of a solution of glyoxylic acid which reacts with tryptophan in the presence of concentrated sulfuric acid to yield a violet-colored product. *Acree and Rosenheim* substituted formaldehyde for the glyoxylic acid and obtained a similar violet coloration. It is believed that each of the three reagents reacts with tryptophan to form a condensation product having the formula:



Xanthoproteic Acid Test. The yellow substance which forms when concentrated nitric acid comes in contact with the skin is the result of nitration of aromatic rings in the skin protein. The color is intensified to a deep orange by treatment with a base. A positive test indicates the presence of phenyl alanine, tyrosine, or tryptophan in the protein.

Ninhydrin Reaction. Although ninhydrin may be used for quantitative determination of free amino acids if the acidity is kept below $pH 4$, it also yields a blue color with proteins under other conditions of acidity. The positive test indicates the presence in the protein of a certain number of free amino and carboxyl groups.

Sulfur Test. The sulfur of a protein is easily set free as inorganic sulfide when the protein is heated with strong sodium hydroxide. Acidification sets free hydrogen sulfide which can be identified with lead acetate paper.

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Study Questions

1. What properties justify grouping together the compounds we call "proteins," in spite of many obvious differences?
2. Give the names and formulas of two dicarboxylic amino acids; of two different basic acids; of five different neutral acids.
3. Contrast the properties of the amino acids with those of the saturated fatty acids in respect to solubility in water and in ether and to physical state. How are these differences explained in terms of structure?
4. Indicate how a neutral amino acid reacts with NaOH; with HCl.
5. Show by a formula why a basic amino acid migrates toward the cathode and a dicarboxylic acid toward the anode. How are these properties used in protein analysis?
6. What is meant by the isoelectric point of an amino acid? How can a water solution of lysine be brought to its isoelectric point? With what would you treat aspartic acid to bring it to its isoelectric point?
7. What analytical use is made of the reaction between an amino acid and nitrous acid? of the reaction between an amino acid and formaldehyde? Write equations for the two reactions.
8. Write the formula for a hexapeptide made up of alanine, glycine, glutamic acid, serine, cysteine, and tyrosine, showing the disposition of the "backbone" in space.
9. Name three protein precipitants; two substances used for "salting out" proteins.
10. How is dialysis used in purification of proteins?
11. How has the ultracentrifuge been used in separation of proteins of a mixture, and how used to determine purity of a protein?

12. What is meant by the statement that a protein sample is "electrophoretically homogeneous"?
13. What is a "conjugated protein"? Name three and indicate the nature of their prosthetic groups.
14. How may hydrolysis of a protein be brought about?
15. How did Stein and Moore separate the amino acids in a protein hydrolysate?
16. What is meant by "paper chromatography"?
17. What is "microbiological assay"?
18. What is believed to be the structure of fibroin? Where is this protein found? Why is it spoken of as a "simple" protein?
19. How does the structure of wool keratin differ from that of fibroin? How do alpha and beta keratin differ from each other? What are current theories as to the structure of alpha keratin? What is the special importance of cysteine in keratin?
20. Give three possible types of protein cross-linkages, and indicate the amino acids involved.
21. What is meant by "denaturation" of a protein? Mention several ways in which this can be brought about. How is denaturation explained in terms of structure?
22. What is glutathione, and what was its special importance in the history of biochemical thought?
23. Where do nucleic acids occur? What products do they yield on hydrolysis?
24. Write the formula for adenine; for cytosine; for 2,4-dioxo-5-methyl pyrimidine.
25. Write the formula for a mononucleotide; for a nucleoside; for adenylic acid; for adenosine.
26. What is now known of the structure of the nucleic acids?

The Lipids

An ordered sequence of chemical reactions in the cell presupposes the work of individual chemical agents and a definite movement of the products, in short a chemical organization. . . . If the morphologist on the one hand investigates the protoplasmic structure, and the biochemist on the other hand tries to assess the chemical processes of the same protoplasm with his cruder but deeper methods, it amounts in the end to two different approaches to the same problem.

FRANZ HOFMEISTER (1931)

The lipids consist of a group of substances which are classified together only because they are all insoluble in water and soluble in the so-called "fat solvents." The chief of these solvents are acetone and ether, ligroin or petroleum ether, chloroform and carbon tetrachloride. The separation of lipids from tissue is usually achieved by some form of continuous extraction, such as the Soxhlet extraction, in which a solvent is allowed to drip repeatedly through the dried and minced tissue. The material which dissolves under these circumstances includes several different kinds of compounds which are roughly classified as follows:

THE LIPIDS

SIMPLE LIPIDS

1. Fats and Oils

Mixed triglycerides of higher fatty acids.

2. Waxes

Esters of higher fatty acids with higher aliphatic alcohols or sterols.

COMPOUND LIPIDS

1. Phospholipids

Glycerol esters of fatty acids and phosphoric acid, with some other hydroxyl compound.

2. Cerebrosides

Compounds of fatty acids, carbohydrates, and a nitrogenous compound.

STEROLS

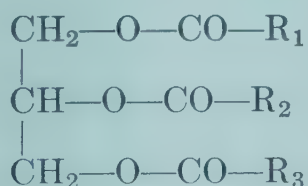
These make up the major part of the "nonsaponifiable" material left as an insoluble residue after the total lipids have been subjected to hydrolysis. They are polycyclic hydroxy compounds related to cyclopentanoperhydrophenanthrene.

Simple Lipids

The "simple" fats consist of a complex mixture of glyceryl esters which yield five to twelve or more different fatty acids when saponified. The mixture is referred to as a fat if it is more or less solid at room temperature, or as an oil if it is liquid. The difference between the two groups is not sharply marked, since it depends on the relative proportions of saturated and unsaturated fatty acids making up the glycerides, and this proportion may be almost infinitely varied. Table 6-I lists some of the common fat acids, with their formulas (p. 214).

GENERAL CHARACTERISTICS OF THE FATS

The physical characteristics of the fats make it very difficult to separate from the complex mixture any one chemical entity. Even the solid fats do not form crystals, and it is impossible to distil them without decomposition. Hence all the early attempts to characterize the fats from different sources had of necessity to be approximations. For example, it is possible to measure the *average* molecular weights, or the *average* degree of unsaturation of the fatty acids in the esters. Somewhat more specific are the analyses in which, after hydrolysis of a fat, the individual fatty acids are at least partially separated and identified. But none of these procedures make it possible to specify the presence in a fat of a single individual chemical compound, since they tell nothing about the actual arrangement of the several fatty acids in triglyceride molecules. Thus until recently there was no way of knowing whether, in a fat sample represented by the type formula given below, the individual molecules



were *simple glycerides*, in which R_1 , R_2 , and R_3 were identical, or *mixed glycerides* in which two or three different acids were esterified with a single glycerol residue. It is now known that the natural fats are mixed esters.

Unsaturation. The figure which represents the degree of unsaturation of a fat is known as its *Iodine Number*. It is defined as the number of grams of iodine which react with 100 g. of fat. Since free iodine does not react readily, the more active iodine bromide (Hanus' Solution) or iodine chloride (Wijs' Solution) is used for the addition reaction, and the iodine equivalent calculated. Pure oleic acid with one double bond has an iodine number of 90, while for clupanodonic acid with five such bonds the number is 384. Therefore, when a sample of butter gives an iodine num-

TABLE 6-I. SOME REPRESENTATIVE ACIDS WHICH OCCUR IN NATURAL FATS AND OILS

SATURATED ACIDS	Formula	Sources
Butyric	$\text{CH}_3-(\text{CH}_2)_2-\text{COOH}$	Butter fat
Isovaleric	$(\text{CH}_3)_2\text{CH}-\text{CH}_2-\text{COOH}$	Porpoise fat
Caproic	$\text{CH}_3-(\text{CH}_2)_4-\text{COOH}$	Butter fat and coconut oil
Caprylic	$\text{CH}_3-(\text{CH}_2)_6-\text{COOH}$	Butter fat and coconut oil
Capric	$\text{CH}_3-(\text{CH}_2)_8-\text{COOH}$	Coconut oil
Lauric	$\text{CH}_3-(\text{CH}_2)_{10}-\text{COOH}$	Coconut oil
Myristic	$\text{CH}_3-(\text{CH}_2)_{12}-\text{COOH}$	Coconut oil
Palmitic	$\text{CH}_3-(\text{CH}_2)_{14}-\text{COOH}$	Animal and vegetable fats
Stearic	$\text{CH}_3-(\text{CH}_2)_{16}-\text{COOH}$	Animal and vegetable fats
Arachidic	$\text{CH}_3-(\text{CH}_2)_{18}-\text{COOH}$	Peanut oil
Cerotic	$\text{CH}_3-(\text{CH}_2)_{24}-\text{COOH}$	Wool fat
UNSATURATED ACIDS		
Palmitoleic	$\text{CH}_3(\text{CH}_2)_5-\text{CH}=\text{CH}-(\text{CH}_2)_7\text{COOH}$	Butter fat
Oleic	$\text{CH}_3(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7\text{COOH}$	All fats
Linoleic	$\text{CH}_3(\text{CH}_2)_4-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_7\text{COOH}$	Linseed oil
Linolenic	$\text{CH}_3-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_7\text{COOH}$	Linseed oil
Arachidonic	$\text{CH}_3(\text{CH}_2)_4-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_3\text{COOH}$	Compound lipids
Erucic	$\text{CH}_3(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_{11}-\text{COOH}$	Rapeseed oil
Ricinoleic	$\text{CH}_3(\text{CH}_2)_5-\text{CHOH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_7\text{COOH}$	Castor oil
Chaulmoogric	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}=\text{CH} \\ \\ \text{CH}-(\text{CH}_2)_{12}\text{COOH} \end{array}$	Chaulmoogra oil
Hydnocarpic	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}=\text{CH} \\ \\ \text{CH}-(\text{CH}_2)_{10}\text{COOH} \end{array}$	Chaulmoogra oil
Tariric	$\text{CH}_3(\text{CH}_2)_{10}-\text{C}=\text{C}-(\text{CH}_2)_4\text{COOH}$	Seed fat of a Central American shrub
Elaeostearic	$\text{CH}_3(\text{CH}_2)_3-\text{CH}=\text{CH}-\text{CH}=\text{CH}-(\text{CH}_2)_7\text{COOH}$	Tung oil
Chupungonic	$\text{C}_{22}\text{H}_{34}\text{O}_2$, having 5 double bonds	Fish oils

ber of about 25, while that for linseed oil is nearer 200, it is clear that the latter mixture includes far more unsaturated acids than the former. In Table 6-II are given figures which indicate the relative degrees of unsaturation of a number of common fats and oils.

TABLE 6-II. DEGREE OF UNSATURATION OF FATS AND OILS

	Iodine Number	Saturated Acids (%) ^a	Unsaturated Acids		
			One Double Bond (%) ^a	Two Double Bonds (%) ^a	Three Double Bonds (%) ^a
Butter	26-38	61.3	32		
Lard	46-66	49	41	6	
Beef tallow	32-47	49	48	3	
Olive oil	80-85	10	83	7	
Cottonseed oil	108	28	19	53	
Tung oil	163-171	3.4	8	10	78
Linseed oil	175-202	10.3	21	15	53
Perilla oil	185-206	6.7	23	—	70

^a Percentages are given as mole per cent, not weight per cent.

Hydrogenation: Understanding of the relation between physical properties and degree of unsaturation has made possible the effective use of great quantities of cheap vegetable oils. As indicated in Table 6-II these are generally characterized by a degree of unsaturation higher than that of the ordinary solid or semisolid animal fats such as butter, lard, and tallow. Hydrogenation of the unsaturated vegetable products can be made to yield fats which resemble lard or butter in physical consistency, and which are suitable substitutes when properly fortified. It is furthermore possible to direct the hydrogenation by means of selective procedures. In cottonseed oil, hydrogen normally adds first to one of the two unsaturated bonds of linoleic acid before making any attack upon the one double bond of oleic acid. Thus without a specific catalyst linoleic acid is hydrogenated four times as fast as oleic. But in the presence of the proper catalyst this difference can be greatly increased so that the ratio of the two rates of hydrogenation becomes 50:1. Furthermore, control of conditions makes it possible to direct the entering hydrogen to a specific one of the two double bonds. Thus saturation of the 9-10 double bond in linoleic acid gives rise to iso-oleic acid, while saturation of the 12-13 unsaturated link results in formation of oleic acid. In this way fats of different compositions and consistencies may be produced at will.

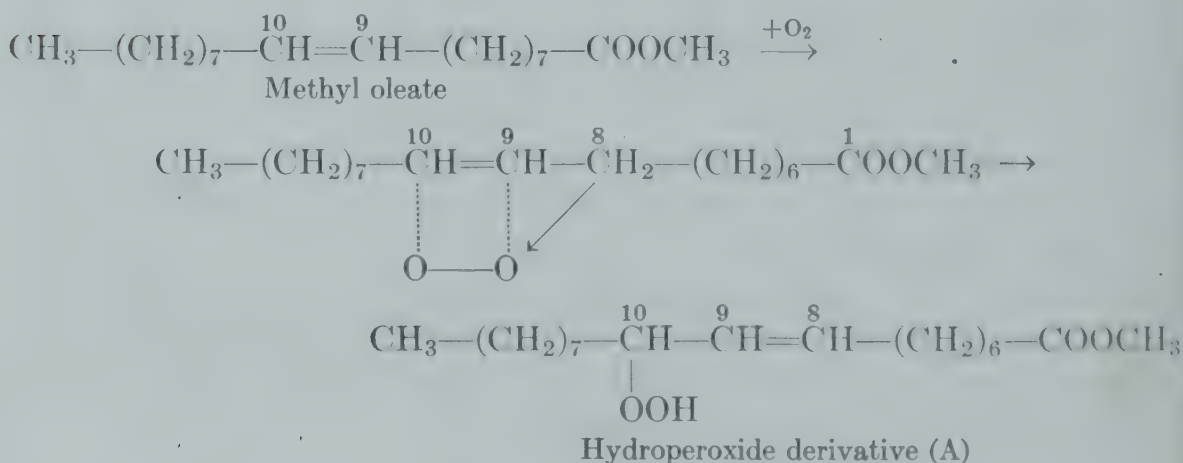
Oxidation of Fats: Associated with the presence of double bonds is the sensitivity of the fats to oxidation. In the drying oils this is a beneficent property of great commercial importance. With food fats the oxidation leads to rancidity, and is a great disadvantage.

The *drying oils* are used in paints and varnishes and in the preparation of oilcloth and linoleum. On exposure to air they form sturdy, insoluble films which resemble the synthetic resins. These are believed to be polymeric products, but the mechanism of their formation and the function of the oxygen are still unknown. One suggestion is that either the oxygen itself or the peroxides which form by addition of oxygen to the double bonds, act catalytically to promote formation of cyclic dimeric and trimeric derivatives. The last three oils listed in Table 6-II, tung oil, linseed oil, and perilla oil, are the best naturally occurring drying oils. The figures indicate not only that these compounds have the high iodine numbers which indicate unsaturation, but also that they include high percentages of acids containing more than one double bond. This is of commercial importance since it appears that the more highly unsaturated acids are first oxidized, and that these oxidation products then act catalytically to promote oxidation of the less' unsaturated molecules.

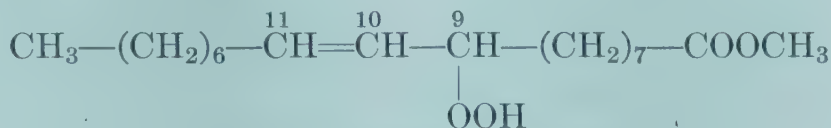
When ordinary fat is kept at room temperature it gradually undergoes changes which give it the unpleasant taste and odor associated with *rancidity*. This condition is in part due to hydrolysis, whereby volatile, bad-odored lower fatty acids are set free. Rancid butter fat for example owes its odor largely to the presence of free butyric acid.

Another process which is undoubtedly involved in rancidity is a slow autoxidation of fatty acid chains, beginning in all probability as an oxidative attack at a double bond. Among the products aldehydes have been identified, as well as ketonic acids, dibasic acids, and fatty acids of reduced chain lengths. The most recent results indicate that in the early stages peroxides are formed, that these then undergo various transformations forming new unsaturated compounds to which a second peroxidic addition of oxygen takes place. It has been suggested that the following preliminary series of reactions is in accord with the known facts and explains the later formation of many known products. In order to simplify the problem, the oxidations have been followed with single pure esters, and the reactions are therefore written for methyl oleate rather than a fat.

1.



Oxygen is first added at the double bond, then by the indicated shift of a hydrogen from carbon 8 (carboxyl carbon being carbon 1) the hydroperoxide derivative forms, with a simultaneous shift of the double bond one position nearer the carboxyl. Obviously the hydrogen which shifts might just as well have come from carbon 11 instead of carbon 8. It is therefore assumed that this first step gives rise to a mixture of the hydroperoxide indicated and of its isomer, which has a Δ^{10} double bond and a hydroperoxide group at carbon 9.

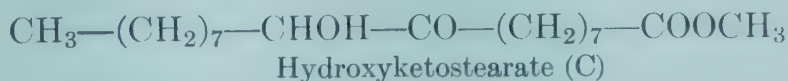
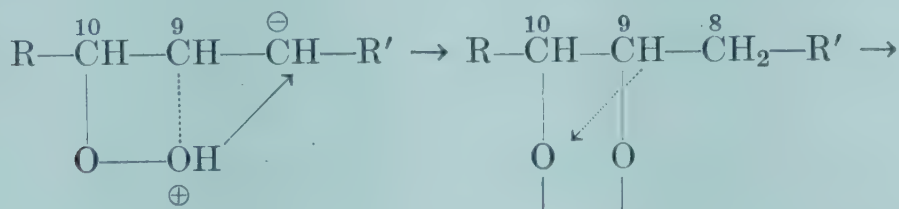
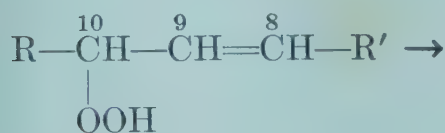


Isomeric Hydroperoxide (B)

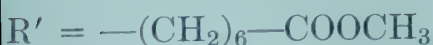
The formulation of the next step shows just one of these isomers (A) assuming that both undergo similar reactions.

2.

There follows a "transition" of the hydroperoxides to hydroxyketostearates.



Hydroxyketostearate (C)



3.

The product indicated above is in equilibrium with the isomer formed from hydroperoxide (B).



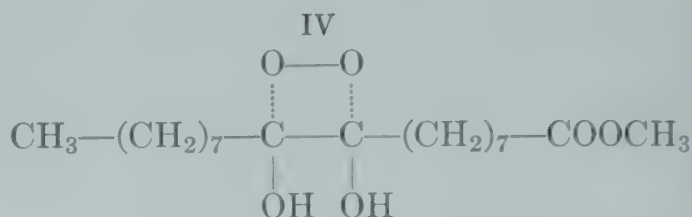
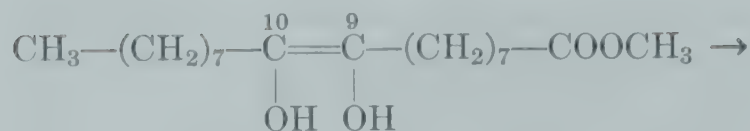
Compound C



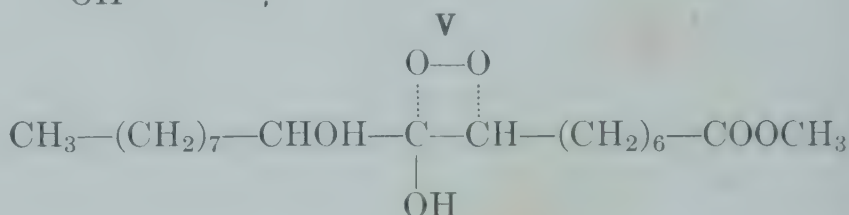
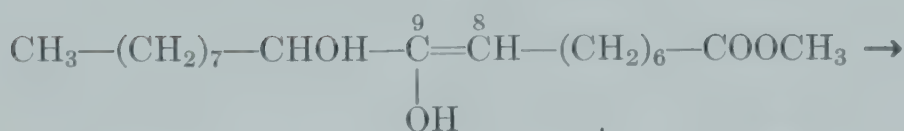
Compound D

These compounds may then by enolization and rearrangement give rise to a mixture of compounds I, II, and III.

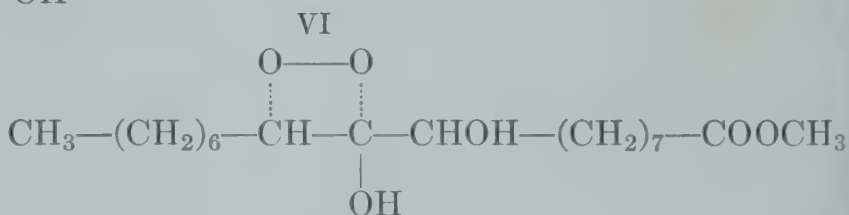
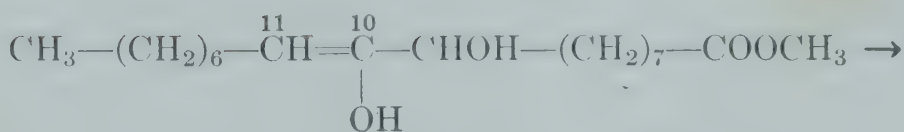
I (Formed from C or D)



II (Formed from C)



III (Formed from D)



The newly formed double bonds are then able to add oxygen again, thus forming for the second time the peroxidic type of compound so characteristic of fats or unsaturated fatty acids in process of autoxidation (compounds IV, V, and VI). The presence of peroxidic oxygen is detected through its ability to set free iodine from potassium iodide. It has been shown that breakdown of these second peroxide compounds would account for many of the known products of autoxidation of fatty acids. For example, nonoic and the dibasic azelaic acid would result from oxidative splitting of compound IV between carbons 9 and 10 while a similar splitting of compound VI would yield octanoic and oxalic acids. These reactions are at this time far from proved, but they are of interest as examples of the types of reactions and compounds which are characteristic of slow oxidation of unsaturated long chain acids.

Some small part of the rancidity of fats is undoubtedly due to microbial action. Certain molds, for example, first hydrolyze the fats, then oxidize the fatty acids to the corresponding ketones. This reaction is believed to begin with formation of a β -ketonic acid, which is then decarboxylated to yield the ketone.



Molecular Weights. Fats differ from each other not only in degree of unsaturation, but in the chain length of the constituent acids. Two procedures are commonly used to determine this characteristic in a general way.

The *Saponification Number* is defined as the number of milligrams of potassium hydroxide required to saponify 1 g. of fat. Obviously, since 1 g. will contain more molecules if the average molecular weight is low, a high saponification number indicates a relatively large number of short chain acids in the molecule. As shown in Table 6-III, the number is higher for

TABLE 6-III. AVERAGE MOLECULAR WEIGHTS OF FATS AND OILS

	Saponification Number	Reichert-Meissl Number
Butter	210-230	17-34.5
Lard	195-203	0.5-0.8
Beef tallow	193-199	0.3
Cottonseed oil	194-196	0.95
Olive oil	185-196	0.6-1.5
Linseed oil	188-195	0.95
Tung oil	190-191	0.35

butter than for most other fats, and this is in agreement with the fact that milk fats contain unusually high percentages of acids with fewer than fourteen carbon atoms.

The *Reichert-Meissl Number* is the number of milliliters of 0.1N alkali required to neutralize the soluble, volatile acids from 5 g. of fat. Of the acids set free by hydrolysis of a fat, only those of short chain length are volatile with steam. A high Reichert-Meissl number is therefore also indicative of a relatively high proportion of acids having a chain of fewer than twelve carbon atoms. The high value for butter differentiates it from other fats and has made it possible to detect its adulteration.

STRUCTURE OF THE FATS

Determination of the actual chemical nature of the fats begins with estimation of the individual fat acids which are present. (The expression "fat acid" is used to distinguish between the mixture of acids found in a fat and the true "fatty acids" which are saturated compounds.) It is

now relatively easy to determine quantitatively the acids present in a given fat since there are available various methods for their separation and identification. The next task, that of assigning structures to the individual triglycerides, is much more difficult, and is indeed only in the preliminary stages.

Separation of Fat Acids. In recent years it has been recognized that part of the tissue lipids occur in association with proteins and that these large complexes are not extracted with the other lipids. In order to obtain complete extraction with fat solvents it is therefore necessary to free the lipids from this association. This is achieved if the dried, minced tissue is extracted for twenty-four to forty-eight hours with methanol. This solvent dissociates the lipoproteins and at the same time extracts the lipid fraction from the tissue very completely. Since it also extracts other cell constituents, the lipids are then isolated by taking the methanol extract to dryness under diminished pressure and extracting the residue with petroleum ether.

In a typical fat analysis the petroleum ether extract is evaporated and the total lipid fraction subjected to saponification. This is brought about by heating the fatty material with an alcoholic solution of potassium hydroxide, in which both fat and reagent are soluble. After distillation of part of the alcohol, the unsaponifiable matter is separated from the water-soluble potassium salts and glycerol by extraction with ether. This fraction consists largely of solid alcohols and sterols. The acids are then set free from their salts by addition of mineral acid, and extracted with ether, leaving the glycerol with the water layer. Steam distillation isolates the group of lower fatty acids, up to and including lauric acid with twelve carbon atoms. The higher acids are then transformed into their lead salts and crystallized from alcohol or ether. This effects the separation of the saturated from the unsaturated acids, the lead salts of the latter being much the more soluble in the organic solvents. After these preliminary steps, the acids in each of the three groups are esterified, and the esters then separated by fractional distillation under diminished pressure. By this procedure most of the acid components of animal and vegetable fats have been identified.

Of recent years other methods of separation have been worked out which bid fair to yield more detailed and complete analyses of these complicated mixtures. Among these are crystallization from organic solvents at very low temperatures, various forms of adsorption analysis, and the use of "countercurrent distribution," in which a mixture is distributed between two solvents in a series of progressive extractions in what amount to micro-separatory funnels.

The Component Acids of Fats. In general the acids known to occur naturally in fats and oils comprise a list of about fifty saturated and un-

saturated acids, among which those with fourteen or more carbon atoms make up by far the largest proportion. With the exception of isovaleric acid $[(\text{CH}_3)_2\text{—CH—CH}_2\text{—COOH}]$ which has been found in the fat of the dolphin and the porpoise, they all contain an even number of carbon atoms, and are nearly all of the straight chain type.

Of all the acids, oleic acid, with its single, central double linkage, is by far the most widespread, making up in some fats 50 per cent of the total fat acids, and appearing in significant amounts in every fat and oil so far examined. Nearly as abundant are two other unsaturated acids, linoleic acid, with eighteen carbon atoms and double bonds at carbons 9 and 12, and palmitoleic acid, with sixteen carbons and a double bond at carbon 9. The saturated acid which occurs most frequently is palmitic acid, $\text{C}_{16}\text{H}_{32}\text{O}_2$, which is almost as ubiquitous as oleic. Myristic ($\text{C}_{14}\text{H}_{28}\text{O}_2$) and stearic ($\text{C}_{18}\text{H}_{36}\text{O}_2$) acids are also widely distributed.

As noted above, fat from a single source contains at least five different acids, and may contain twelve or more. Of these there are usually only two or three which are present to the extent of 10 per cent or more. Hilditch¹ refers to these as "major constituents" as opposed to the "minor" acids, each of which makes up less than 10 per cent of the total mixture.

Since it has become possible to survey broadly the composition of many plant and animal fats, a pattern begins to be evident. Except for a very few plant oils in which there may occur an acid peculiar to a given plant or to its species, the acids which make up the fats all come from a relatively short list. The same acids occur again and again in animal, fish, and plant fats, the different properties of which depend quite as much on differences in relative proportions as on actual differences in component acids. In general, the fats of the simplest and most primitive organisms are the most complex in composition while those of the higher forms contain fewer different acids. Furthermore, the fats from organisms which have been grouped together by the biologists or the botanists are often found to share the same acids as major constituents. Thus in aquatic forms the unsaturated acids predominate, with those containing sixteen, eighteen, twenty, and twenty-two carbon atoms making up the major part. Among these aquatic fats, there is a distinction to be drawn between those of marine organisms and those of fresh-water forms, the latter showing a tendency to utilize the C_{16} and C_{18} unsaturated acids while the marine fats include higher proportions of the C_{20} and especially the C_{22} unsaturated acids. The body fat of the salmon alters progressively in conformity with this generalization as the fish go from their breeding grounds in fresh water out to the open sea.

¹ Thomas P. Hilditch (1886—) is Professor of Industrial Chemistry at the University of Liverpool. He and his associates are responsible for many of the modern methods of fat analysis, and have used these methods to amass an enormous volume of information about the constitution of natural fats.

The depot fats of land animals are on the whole simpler than those of aquatic origin, and include higher proportions of saturated acids and much smaller amounts of the very long chain unsaturated ones. Some of these differences are illustrated in Table 6-IV, the data for which all come from Hilditch's book.

TABLE 6-IV. COMPONENT ACIDS IN DEPOT FATS OF ANIMALS ON NATURAL DIETS ^a

	Saturated	Unsaturated			
	Palmitic Acid (%) ^b	C ₁₆ (%) ^b	C ₁₈ (%) ^b	C ₂₀ (%) ^b	C ₂₂ (%) ^b
Fish, fresh-water	13-15	ca. 20	40-45	ca. 12	0-5
Fish, marine	12-15	15-18	27-30	20-25	8-12
Frog	11	15	52	15	
Lizard	18	10	56	5	
Rat	24-28	7-8	ca. 60	0.3-0.5	
Cat	29	4	43	Trace	
Ox	27-30	2-3	40-50	0.2-0.5	
Lion	29	2	40	3	
Man	24-25	5-7	53-57	2-2.5	

^a Data from T. P. Hilditch, *Chemical Constitution of Natural Fats*, 1947.

^b Percentages are weight percentages.

Among the plants the same progression is apparent, with the more primitive plants elaborating the more complex fats. Here the same acids appear as in animal fats, their distribution seeming often to follow family lines. The pattern is, however, varied to this extent, that in certain species the seed fats contain a high percentage of an acid which is not otherwise known as a fat constituent of that plant. Outstanding examples are the cyclic acids, chaulmoogric and hydnocarpic acids, found in chaulmoogra oil. This oil is the product of a thorny shrub which grows in tropical Asia and Africa and belongs to the *Flacourtiaceae*. In the East the oil was used medicinally for leprosy for generations before modern medicine discovered its efficacy. Other acids which are narrowly distributed are the acetylenic tariric acid, which is found in the *Simarubaceae*, eleostearic acid with its three double bonds which occurs almost uniquely in tung oil, and ricinoleic acid which constitutes about 85 per cent of the acid in castor oil.

It is perhaps worth noting that in spite of the uniformities, the biosynthesis of fat by a given organism exhibits also a rather surprising variety. Among the marine mammalia and the more primitive land animals, milk and depot fats have similar compositions, but in the higher land animals there is a distinct difference, the milk fats including a far higher percentage of the lower saturated acids. This we have already noted in butter fat. Similarly among the plants, in some species the fruit and seed fats are alike, but in others their compositions differ widely.

TABLE 6-V. COMPARISON OF DIFFERENT FATS ELABORATED BY THE SAME ORGANISM ^a

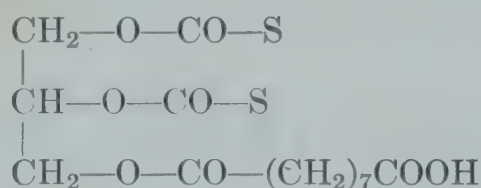
Organism	Tissue	Component Acids: Mol. per cent of Total						
		Saturated Acids				Unsaturated Acids		
		Acids with less than 12 carbons	Lauric acid C ₁₂	Myristic acid C ₁₄	Palmitic acid C ₁₆	Stearic acid C ₁₈	Oleic acid 1 D.B.	Linoleic acid 2 D.B.
								Unsatur. C ₂₀ and C ₂₂ acids
Palm tree	Fruit	—	—	—	35-40	—	40-50	5-11
	Seed	—	47	14	9	—	18	1
Olive tree	Fruit	—	7-15	—	70-85	—	4-12	—
	Seed	—	—	—	6	4	83	7
Cacao tree	Fruit	—	—	—	50	—	35	10
	Seed	—	—	—	24	35	38	2
Sheep	Depot fat	—	—	3.0	28	16.2	46.6	3.9
	Milk	22.7	4.5	9.9	21.6	10.3	21.6	4.3
Human	Depot fat	—	0.1	2.7	24	8.4	46.9	10.2
	Milk	2.5	8.3	8.7	22.8	8.3	33.9	7.7
								0.6 + 0.8 lower unsat. acids ^b
								1.3 + 3.0 lower unsat. acids ^b
								2.5 + 5.2 lower unsat. acids ^b
								2.4 + 4.6 lower unsat. acids ^b

^a Data from T. P. Hilditch, *Chemical Constitution of Natural Fats*, 1947.

^b Includes unsaturated acids from C₁₀ through C₁₆.

There was once current a theory that temperature determined the type of fat elaborated by an organism. Clearly this cannot be a decisive influence if the same organism synthesizes fats of quite different composition to serve different purposes. Table 6-V gives figures which illustrate these differences in a few representative organisms.

The Component Triglycerides of Fats. Our knowledge of the actual composition of natural triglycerides is quite recent and depends largely upon methods elaborated by Hilditch and his colleagues. In one procedure the fully saturated triglycerides are separated from those which contain one or more unsaturated acids by first subjecting the fat to oxidation with potassium permanganate in acetone solution. This splits the carbon chain at the double linkages, forming two carboxyl groups, and gives rise as chief product to acidic compounds which are known as "azelao-glycerides," since one or more of the acid residues are now those of azelaic acid, $\text{HOOC}-(\text{CH}_2)_7-\text{COOH}$. This results from the fact that many unsaturated fat acids have seven CH_2- groups between the double link and the esterified carboxyl group. Thus a triglyceride in which two saturated (S) acids plus oleic acid made up the triglyceride would, on oxidation, give the monoazelao-glyceride below:



Having now a free carboxyl group, this compound is soluble in potassium hydroxide. By extracting with alkali, the potassium salts of all the azelao-glycerides may be separated from those which were fully saturated and which were therefore not attacked by permanganate at all. The potassium salts are then fractionated, making use of the lower solubility in ether of those having only one carboxyl group. Given such quantitative data as may be obtained in this sort of estimation, plus a knowledge of the actual component acids of a fat, it is possible to calculate at least the distribution of saturated and unsaturated acids in the mixed triglycerides.

It was found, rather surprisingly, that even when saturated acids make up 25 per cent or more of the total, the percentage of fully saturated *triglycerides* is very small. This can only mean that the saturated acids present are distributed among many triglyceride molecules, and that each triglyceride contains some unsaturated acid. In the early days of fat chemistry the simple triglycerides were named from the acid involved, as tristearin (or even "stearin"), triolein, etc. Modern work makes it clear that such molecules, if present at all, make up only a very small fraction of the total mixture. It is also generally accepted that the arrangement of acids in triglycerides is not a result of random distribution. Hilditch

sums up his conclusions in these words, "Apparently the fatty acids are interwoven with the glycerol molecules so as to avoid where possible approach to triglycerides containing identical acyl radicals, but concurrently the mixed glycerides are not produced indiscriminately, but are given preferred configurations." Hilditch favors the theory of "even distribution," according to which each acid is as widely distributed as possible and appears twice in a triglyceride molecule only when it makes up more than 33 per cent of the total acids.

Other patterns of distribution have been suggested, modifying somewhat the rigidity of the Hilditch formulation. Until many more data are available it will be impossible to map exactly the glyceride composition of any fat, but the balance of evidence at the moment points to a wide dispersal of each acid among the various triglycerides. In Table 6-VI are given

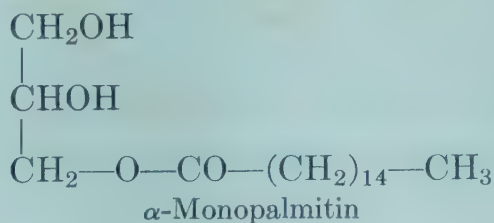
TABLE 6-VI. TRIGLYCERIDE COMPOSITION OF FATS AND OILS ^a

	Cocoa Butter (mol. %)	Shea Butter (mol. %)	Butter (mol. %)	Ox Depot Fat (mol. %)
Trisaturated glycerides	2.5	2.3	34	17
Disaturated-mono-unsaturated glycerides	77	30-65	37-51	49
Monosaturated-diunsaturated glycerides	16	70-0	30-0	34
Triunsaturated glycerides	4	0-35	0-15	Trace

^a The data in this table have been adapted from figures obtained by Hilditch and his associates and quoted by Longenecker, H. E., *Chem. Revs.*, 29:201, 1941.

examples of the type of data now beginning to be available about the triglyceride composition of fats.

A Glycerol Monoester. Quite recently there has been isolated from hog pancreas a white, waxy, crystalline substance which proved to be α -monopalmitin, present in pancreas to the extent of nearly 2 per cent.



This compound is apparently quite sharply localized as none was found in brain, adrenal gland, or liver.

WAXES

Although some of the waxes are of commercial importance, they do not seem to play any active role biologically. They are sometimes referred

to as "end products" of plant metabolism. They occur coating the cuticle of leaves and on the surfaces of seeds and fruit. They also make up a fraction of whale blubber and of fish liver fats, and are elaborated by various insects and bacilli.

Several different alcohols and acids have been separated from the waxes but few of them have been obtained in the pure state. In general the waxes consist of esters of even-numbered fatty acids with sterols or with monohydric aliphatic alcohols of high molecular weight. These esters are usually found mixed with varying amounts of paraffin hydrocarbons and with free alcohols or acids. The acids and alcohols are even-numbered ones containing twenty-six to thirty-four carbon atoms, while the paraffins are odd-numbered compounds with twenty-five to thirty-seven carbons. A few plant waxes have been shown to contain only a single alcohol, but usually the waxes are complex mixtures, none of which have yet been completely fractionated. The major components of a few common waxes are listed in Table 6-VII.

TABLE 6-VII. MAJOR COMPONENTS OF COMMON WAXES

Wax	Chief Ester	Formula
Carnauba wax	Myricyl cerotate ^a	$C_{25}H_{51}COOC_{30}H_{61}$
Beeswax	Myricyl palmitate	$C_{15}H_{31}COOC_{30}H_{61}$
Chinese wax	Ceryl cerotate	$C_{25}H_{51}COOC_{26}H_{53}$
Opium wax	Ceryl palmitate	$C_{15}H_{31}COOC_{26}H_{53}$

^a Both the acids and the alcohols of the esters in the list are saturated.

Compound Lipids

The pioneer work with the compound lipids was done by Thudicum ² who named these fatlike substances, most of which contain both nitrogen and phosphorus, the "phosphatides." They are extracted from tissue with the fats and are then separated from the fats and from each other by differences in solubility in such solvents as alcohol, acetone, and ether. It is now known that some members of the group of compound lipids do not contain a phosphoric acid residue, and so the name phosphatide to designate the group has been gradually dropped, and the phosphorus-containing members of the group are now known as *phospholipids*.

PHOSPHOLIPIDS

It was at first believed that there were just three phospholipids, lecithin, cephalin, and sphingomyelin. The first and third still retain some measure

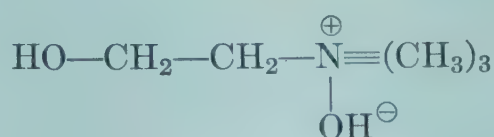
² Johann L. W. Thudicum (1829-1921) was born in Germany and took his medical degree there, but spent most of his professional life in London, first at St. George's School of Medicine, and later as Pathologist at St. Thomas' Hospital. He is chiefly remembered for his pioneer work on the chemical composition of the brain.

of identity, but the second has proved to include at least three different substances. It will perhaps be simplest to consider lecithin first, and then to compare the other compounds with it.

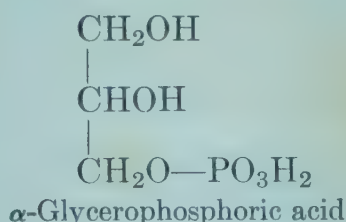
Lecithin. Lecithin, or rather the lecithins, occur mixed with other phospholipids in all animal and vegetable cells, but are present in higher concentrations in brain, heart, kidney, and eggs among animal tissues, and in plant seeds. Soybean lecithin is an important article of commerce, being widely used as an emulsifier.

Lecithin is a yellowish-white waxy solid, soluble in hot alcohol, from which it can be precipitated either with acetone or by addition of cadmium chloride.

On hydrolysis with barium hydroxide lecithin yields a mixture of glycerophosphoric acid, fat acids, and the strongly basic *choline*. Choline plays an important part in the regulation of fat metabolism in animals, and is involved, in the form of acetyl choline, in the transmission of nerve impulses.

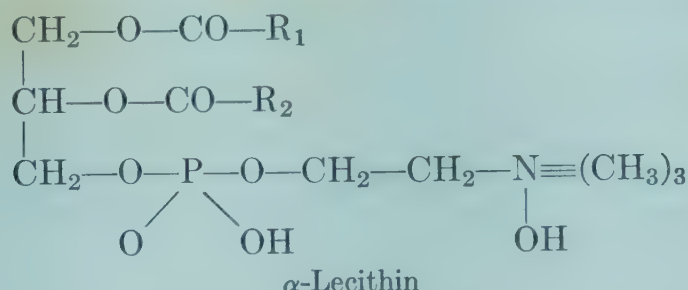


Choline
Trimethyl- β -hydroxyethyl-
ammonium hydroxide



α -Glycerophosphoric acid

The structure of lecithin was formulated many years ago, as follows:



The R_1 and R_2 represent fat acid residues and since at least five different acids are obtained from even highly purified lecithin, the latter must be a mixture of several different glycerides. In general the acids from lecithin are highly unsaturated.

The β -form of lecithin would have the phosphorylcholine grouping on the central carbon of the glycerol. It was believed for many years that the natural lecithins consisted of mixed α - and β -forms, since both α - and β -glycerophosphoric acid were isolated from the hydrolytic mixture. Recently it has been shown, however, that pure synthetic α -lecithin gives rise to just such a mixture when it is hydrolyzed. Clearly, therefore, the phosphoryl residue may shift its position under the conditions of hydrolysis, and " β -lecithin" is probably an artifact.

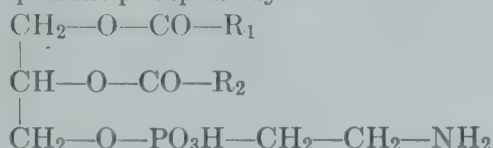
Other Phospholipids. The early workers separated from the mixed phospholipids extracted from brain a fraction insoluble in alcohol to which they gave the name *cephalin*. The structure assigned was that of a compound exactly like lecithin except that ethanolamine, $\text{HO}-\text{CH}_2-\text{CH}_2-\text{NH}_2$, replaced the choline. This fraction has recently been shown to consist of at least three different compounds, of which one has the structure originally assigned to cephalin. In a second compound the amino acid serine, attached through its hydroxyl group, replaces the ethanolamine, but the third is a totally different type of compound made up of glycerol, fat acids, and phosphoric acid combined with the cyclic hexahydric alcohol, inositol. As a result of this fractionation an amplified classification of the compound lipids has been proposed and is outlined in Table 6-VIII. In connection

TABLE 6-VIII. RECLASSIFICATION OF THE COMPOUND LIPIDS (PHOSPHOLIPIDS)

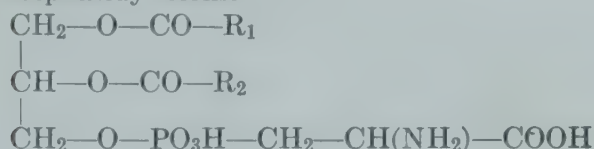
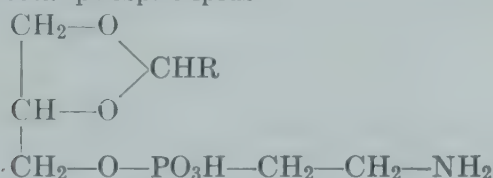
1. Phosphoglycerides

a. Lecithin: diacylglycerophosphoryl choline or phosphatidyl choline.

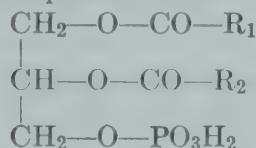
b. Cephalin: phosphatidyl ethanolamine



c. Phosphatidyl serine

d. Acetal phospholipids ^a

e. Phosphatidic acids



2. Phosphoinositides

Compounds which contain inositol in addition to glycerol, fat acids, and phosphoric acid.

3. Phosphosphingosides

Compounds in which the dihydroxyamine, sphingosine, serves as glycerol does in the glycerides, to join fat acid, phosphoric acid, and base residues.

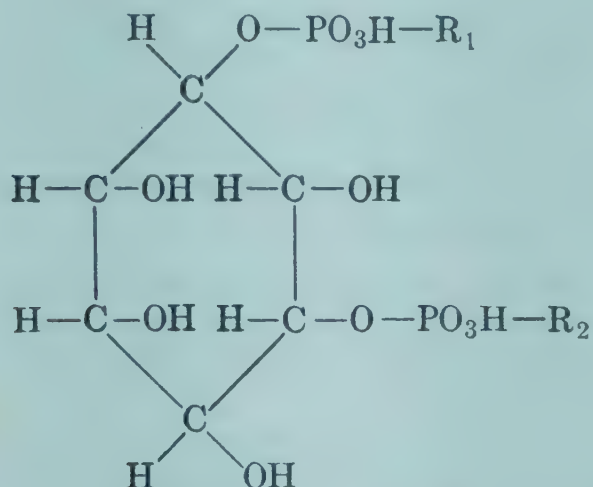
^a These compounds are also known as plasmalogens.

with the names indicated, it should be noted that a compound constituted like lecithin but lacking choline, i.e., diacylglycerophosphoric acid, is also known as a "phosphatidic acid."

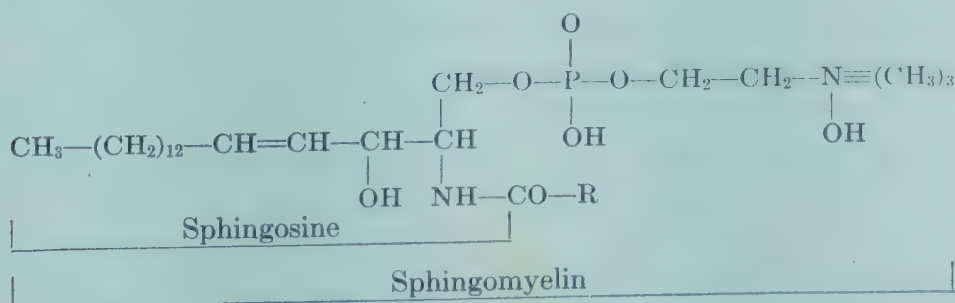
The structures noted in the outline are self-explanatory as far as the phosphoglycerides are concerned. In the acetal compound, two of the hydroxyl groups of glycerol have condensed with an aldehyde related to

one or other of the higher fatty acids. It is perhaps unnecessary to have included the fifth group, of the phosphatidic acids, though those substances have been believed to occur free in plants. It has recently been found that some plants elaborate an enzyme capable of splitting choline from lecithin, and if such enzymes are at all widely distributed they may cause formation of phosphatidic acid from plant lecithin under the conditions of extraction.

The newly discovered *phosphoinositides* correspond to those plant products which have been known as "lipositols." Lipids which include inositol have been isolated from plants, from brain tissue, and from bacteria, but in no case has the structure been completely worked out. The compound from brain yields on hydrolysis equimolecular amounts of fat acid, glycerol, and inositol metadiphosphate. The following structure has been suggested for part of the molecule, with the residues, R_1 and R_2 not identified.



Although only one substance, *sphingomyelin*, has yet been identified, it is probable that the *phosphosphingosides* constitute a mixture of similar substances yielding on hydrolysis fat acids, phosphoric acid and two bases. One of these bases is choline and the other either sphingosine or a closely related base. It is only recently that the structure of sphingosine has been completely elucidated so that it is now possible to assign the following structure to the main product in the phosphosphingoside fraction. In the sphingoside the amino group is bound to an acyl group through a peptide linkage, and the terminal hydroxyl group holds a phosphocholine residue.



There has also recently been found in the phosphosphingoside fraction from both brain and spleen a third base, dihydrosphingosine. This, with

the fact that hydrolysis always yields a mixture of acids, makes it likely that there are a number of closely related phosphosphingosides.

CEREBROSIDES

Although they occur in many cells of the animal body and have been found in some plant tissues, the group of compounds known as the *cerebro-*sides are particularly abundant in nervous tissue. They differ from most lipids in being insoluble in ether and petroleum ether as well as in water, but they dissolve in warm alcohol which is therefore used to extract them from tissue. The cerebroside were discovered and named about 1874 by Thudicum, but they are so very difficult to work with that our information about them is still very incomplete.

On hydrolysis the cerebroside yield the base sphingosine, a fat acid of high molecular weight, and a hexose. At one time it was believed that the sugar was always galactose, but there is now evidence that glucose also forms similar compounds. Four compounds of this general composition have been sufficiently characterized to receive names. These are cerebron (phrenosin), kerasin, nervon, and oxynervon. They are believed to share the common building plan indicated below, differing in their acid and possibly in their sugar constituents.



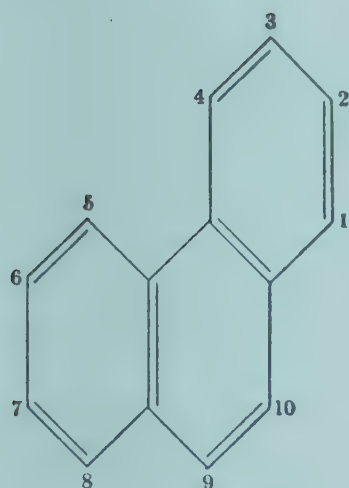
The acid is presumably linked to the base as an amide, and the sugar to one of its two hydroxyl groups.

The acids which have been isolated from hydrolyzed cerebroside are long chain compounds and include the following C_{24} -acids all of which are closely related to each other:

$\text{CH}_3 \text{---} (\text{CH}_2)_{22} \text{---} \text{COOH}$	Lignoceric acid
$\text{CH}_3 \text{---} (\text{CH}_2)_{21} \text{---} \text{CHOH} \text{---} \text{COOH}$	Cerebronic acid
$\text{CH}_3 \text{---} (\text{CH}_2)_7 \text{---} \text{CH}=\text{CH} \text{---} (\text{CH}_2)_{13} \text{---} \text{COOH}$	Nervonic acid
$\text{CH}_3 \text{---} (\text{CH}_2)_7 \text{---} \text{CH}=\text{CH} \text{---} (\text{CH}_2)_{12} \text{---} \text{CHOH} \text{---} \text{COOH}$	Oxynervonic acid

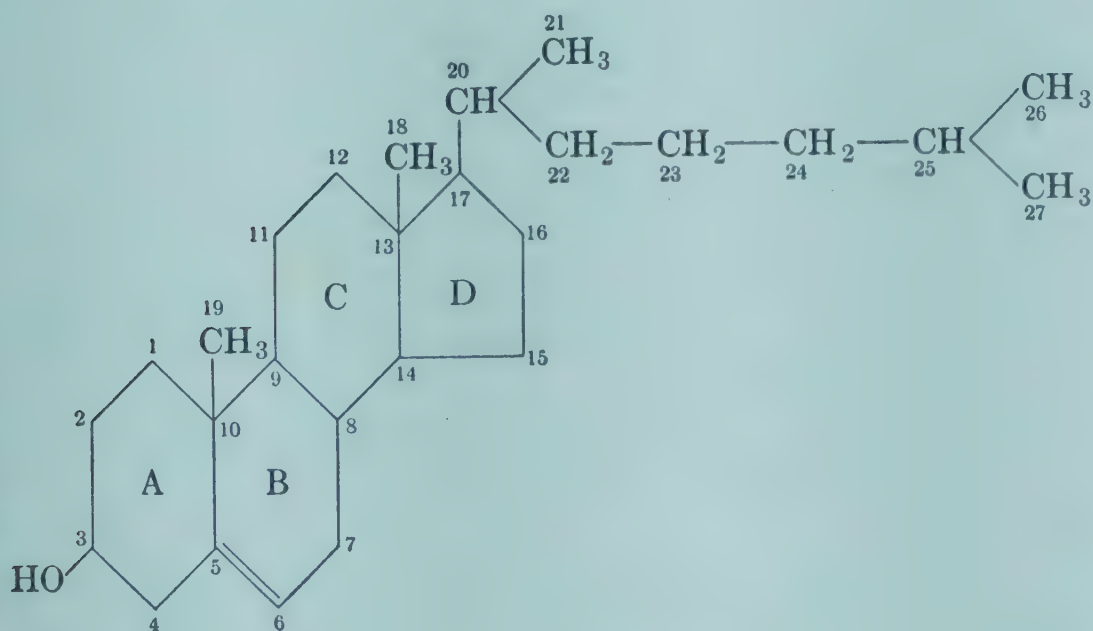
The Sterols

Besides such readily hydrolyzable compounds as have just been considered, the fat solvents extract from plant and animal tissue a certain amount of material which does not react with alkali to form soluble compounds. This nonsaponifiable matter includes any long chain aliphatic alcohols such as are found in the waxes, and also the group of substances known as *sterols* or "solid alcohols." These latter are crystalline cyclic alcohols which occur both free and esterified with long chain fatty acids. They all contain a saturated phenanthrene ring system to which is fused at the 1,2 positions an additional five-membered ring.



Phenanthrene

The first of this group of compounds to be recognized was the chief animal sterol, cholesterol, first isolated from gallstones in 1775. It is present both free and esterified, in the blood and in all animal cells. The structure of cholesterol is indicated below, as well as the somewhat irrational numbering system which has grown up as the structure of these compounds has been slowly elucidated. The rings indicated are not aromatic rings, but are fully saturated except where a double bond is shown. The compound is therefore an hydroxyl derivative of a substituted perhydro-1,2-cyclopentanophenanthrene. The presence of methyl groups at positions 10 and 13 is characteristic of most of the natural compounds related to cholesterol, as is some sort of substituent at position 17. As indicated, the rings are often identified by letter. Note that the numbering of the sterols does not follow that of phenanthrene, and so the 1,2 positions in phenanthrene become 14,13 in cholesterol.



Cholesterol

Many biologically active compounds have proved to be closely related in structure to cholesterol. The sex hormones, both male and female, the main constituent of the bile acids (see p. 325), and the steroid hormones of the adrenal cortex are among the animal products having very similar structures. The sterols of plant origin are known as *phytosterols* and include sitosterol and ergosterol, the latter yielding the closely related vitamin D on irradiation with ultraviolet light.

Other plant products having both this same type of structure and a pronounced physiological activity are the so-called cardiac glycosides. These substances are found in the seeds and leaves of the purple foxglove from which they are extracted to yield the medicinal "digitalis." This substance consists of glycosides in which various simple sugars are condensed with ring compounds closely related to cholesterol.

The chemistry and stereochemistry of all this group of compounds is complex in the extreme, and cannot be considered in detail here. For a discussion of the isolation, purification, and properties of the sterols the reader is referred to the *Chemistry of Natural Products Related to Phenanthrene* by Fieser and Fieser.

Suggestions for Further Reading

GENERAL

The following books are noted because they contain numerous references to various phases of the subject of lipid chemistry.

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BULL, H. B., *The Biochemistry of the Lipids*, Wiley, New York, 1937.

FIESER, L. F., and FIESER, M., *Organic Chemistry*, Heath, Boston, 1950, Chapters 16 and 39.

FIESER, L. F., and FIESER, M., *Chemistry of Natural Products Related to Phenanthrene*, 3rd ed., Reinhold, New York, 1949.

HILDITCH, T. P., *The Chemical Constitution of the Natural Fats*, 2nd ed., Wiley, New York, 1947.

This is the standard and comprehensive book on the subject.

MARKLEY, K. S., *Fatty Acids, Their Chemistry and Physical Properties*, Interscience, New York, 1947.

RALSTON, A. W., *Fatty Acids and Their Derivatives*, Wiley, New York, 1948.

The Annual Review of Biochemistry carries each year an article summarizing the recent results in lipid chemistry.

"Symposium on the Molecular Structure of Fats and Oils," *Chem. Revs.*, 29:199, 1941.

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CHIBNALL, A. C., PIPER, S. H., POLLARD, A., WILLIAMS, E. F., and SAHAI, P. N., "Constitution of the Primary Alcohols, Fatty Acids and Paraffins Present in Plant and Insect Waxes," *B.J.*, 28:2189, 1934.

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- SKELLON, J. H., "The Mechanism of Oxidation of Monoethenoid Fatty Acids," *J.C.S.* (1950), 2020.

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- BAER, E., and KATER, M., "Migration During Hydrolysis of Esters of Glycerophosphoric Acid," *J.B.C.*, 175:79, 1948.
- CARTER, H. E., GLICK, F. J., NORRIS, W. P., and PHILLIPS, G. E., "Structure of Sphingosine," *J.B.C.*, 170:285, 1947.
- FOLCH, J., "Complete Fractionation of Brain Cephalin," *J.B.C.*, 177:497, 1949.
- "Brain Diphosphoinositide," *J.B.C.*, 177:505, 1949.
- THANNHAUSER, S. J., BONCODDO, N. F., and SCHMIDT, G., "Studies of Acetal Phospholipids of Brain," *J.B.C.*, 188:417, 1951.

Study Questions

1. Give the names and formulas for: two saturated fatty acids of common occurrence; an unsaturated acid.
2. Name three fat acids which contain more than one double bond.
3. What is measured by the Iodine Number of a compound? by the Saponification Number of a fat? What is known of the structure of a fat which has a high Saponification Number?
4. Name three different causes which contribute to rancidity of a fat.
5. Which is the most widely distributed fatty acid?
6. Would you expect the Iodine Number of a plant oil to be higher or lower than that of an animal fat?
7. What is known of the composition of individual triglycerides?
8. How is the composition of its fat related to the place of an organism in the evolutionary scale?
9. To what class of compounds does phosphatidyl choline belong? What is the common name of this compound? its formula?
10. How do phosphatidyl ethanolamine and phosphatidyl serine differ from lecithin?
11. Write the formula for sphingosine and indicate the structure of sphingomyelin.
12. Write the basic ring structure of the sterols and number the carbons. At which position has cholesterol an —OH group? a double bond? Where are its side chains?

The Enzymes

Science is a wonderful exhibition of stages in the greatest of human adventures, the intellectual adventure.

J. W. N. SULLIVAN: *The Limitations of Science* (1933)

It was obvious from early biochemical studies that part of the foodstuffs give rise to carbon dioxide and water; that nitrogen ingested in the form of protein is largely excreted as urea. It has been one of the main goals of biochemistry to discover the steps by which such changes as these are brought about. Through what series of transformations is a complex polysaccharide degraded to so simple a substance as carbon dioxide? How is the nitrogen locked up in protein linkages first set free, and then synthesized into the small urea molecule? It is the purpose of this chapter to consider the agents which catalyze the many chemical reactions taking place in living cells.

It is a familiar fact that if bacteria and molds are excluded, solutions of sucrose or glucose are perfectly stable and can be kept unchanged for months. Yet both, if introduced into a living organism, undergo rapid chemical transformation. Polar explorers report that within twenty minutes the heating effect of an ingested chocolate bar can be noted with a thermometer. This means that within so brief a time sucrose has been hydrolyzed, the split products have been absorbed, and at least some of them have been oxidized. Outside of the body this hydrolysis and oxidation could be brought about only under conditions of temperature or acidity which would be lethal to living tissue. The agents which give to living organisms the ability to digest, to oxidize, and to carry out a host of other chemical reactions are the catalysts or *enzymes* which they elaborate. Those enzymes which are secreted into the digestive tract act outside the cells, but a far greater number carry out their functions intracellularly.

Early Work on Enzymes

Although fermentation of grape juice and the making of cheese and souring of milk had been known from very early times, the study of the agents concerned in such catalytic processes goes back only to the early years of the nineteenth century. In 1830 Robiquet and Boutron-Charlard obtained by distillation of bitter almonds the fragrant "volatile oil" which we call

benzaldehyde. In a study of the properties of this substance and of its relationship to the inert, odorless "fixed oil" which they obtained if they simply expressed the juice from the kernels by pressure in the cold, they were puzzled by the apparently unpredictable appearance of the fragrant substance. They decide that "quelques corps occulte" must be involved but made no attempt to identify it.

A few years later Wöhler and Liebig¹ solved the mystery by showing that the liquid obtained from bitter almonds by pressure contains a glucoside, amygdalin. At an elevated temperature this substance hydrolyzes readily to yield glucose, benzaldehyde, and hydrogen cyanide. They proved that the "vegetable proteins" of the almond itself, left as a residue after extraction of amygdalin by pressure, contained some substance which greatly hastened hydrolysis of the glucoside at room temperature. They named the active agent "emulsin" because it was found in the emulsion formed when the protein residue was extracted with water. In the light of his results with the extract Liebig wrote: "The small amount of emulsin which is necessary . . . to bring about the breakdown of amygdalin . . . shows that we are dealing here with no ordinary chemical reaction; there is a definite resemblance to the action of yeast on sugar, which Berzelius describes as catalytic." Even in these early papers Liebig foresaw the possibility that there were in organic nature other agents of a similar nature, "each concerned with the preparation of a special substance." Later chapters will show how good a prophet he was, for the economy of living cells, whether plant or animal, is mediated and controlled by a whole battery of such catalytic agents, each of which fulfills a unique function.

For many years there was no uniformity in the nomenclature of these new organic catalysts. Malt diastase, found in sprouting barley, was one of the first of these agents to be precipitated from solution, and as a result many writers, especially in France, refer to all such active agents as *diastases*. Pasteur (1822–1895) who did much of the pioneer work on alcoholic fermentation, used the word *ferment* to designate the entire group of catalysts, and in Germany the use of that name has persisted. But in English-speaking countries the name which has come into general use is "enzyme," first proposed by Willy Kühne in 1878. The substance on which an enzyme exerts its catalytic effect is known as a *substrate*, and many individual enzymes are now named by adding *-ase* to the name, or part of the name, of the substrate. *Urease* is thus the enzyme which brings about hydrolysis of urea, and *sucrase* catalyzes the inversion of sucrose.

¹ Justus von Liebig (1803–1873) and Friedrich Wöhler (1800–1882) were friends and research associates for many years, Wöhler often attempting though with scant success to stem the tide of his colleague's scientific polemics. It would be hard to overestimate the debt which organic chemistry owes them for the long series of brilliant researches which they carried out, sometimes in collaboration and sometimes working separately. Wöhler was Professor at Göttingen and Liebig first at Giessen and later at Munich.

Although the next few years following the publication of Wöhler's and Liebig's papers on emulsin saw the identification of many enzymes of plant and animal origin, the chief interest of scientists centered in the studies of the nature of alcoholic fermentation. As early as 1837 Berzelius had recognized the catalytic nature of the reaction, but the names chiefly associated with the early studies are those of Liebig and Pasteur. For many years these two eminent scientists engaged in a running battle over the nature of enzyme action. Pasteur's views were colored by his own microscopic study of yeasts, while Liebig was thinking in terms of the enzyme he knew best, emulsin.

Pasteur had proved that such processes as alcoholic fermentation and the souring of milk and wine took place only in the presence of certain definite microorganisms. He knew that the organisms were alive, for he found that they multiplied rapidly while exerting their catalytic activity. He deduced from this that the chemical changes taking place were inseparable from the simultaneous vital activities of the organisms involved. Liebig, on the other hand, had seen and indeed taken a leading part in the triumphant advances in organic chemistry which followed the overthrow of the old "vital force" theory. He was therefore understandably averse to allowing a similar theory to come to the fore in the field of enzyme chemistry. His emulsin was certainly not a product of a reproducing microorganism, and he therefore argued for a purely chemical explanation of all enzyme activity. He pictured a stable molecule as one in which the "amplitude of vibration" of its atoms does not exceed a certain amount. He argued that if a substance, itself unstable and therefore decomposing, were brought into contact with a more stable one it would so upset the vibration equilibrium of the stable molecule as to cause it to begin to decompose also. He thought emulsin acted upon amygdalin because the vegetable proteins were decomposing, and he insisted that yeast acted upon sugar because the yeast, or some part of it, was also undergoing putrefaction. In just these terms, Liebig's theory is of course untenable. But it will be worth while to consider it again a little later. It will be found that modern ideas of enzyme action are not so very far from Liebig's, though couched now in more precise terms.

Liebig had been dead for twenty-four years and Pasteur for two when, in 1897, Eduard Buchner² settled the dispute in Liebig's favor. He showed that yeast cells, ruptured by grinding with sand, yield under high pressure a juice which, though entirely cell-free, yet brings about the fermentation of sugar. This experiment opened a whole new field in enzyme chemistry. Once it had been shown that one catalyst could be separated in solution

² Eduard Buchner (1860-1917) was Professor of Chemistry at Tübingen when he separated the yeast enzymes from the cells. He was awarded the Nobel Prize in Chemistry in 1907. In 1911 he went to Würzburg as Director of the Chemical Institute of the University, but was on war duty at the time of his death.

from the cells which elaborated it, it was inevitable that other micro-organisms as well as plant and animal tissues should in turn be subjected to the action of solvents. Certain enzymes, of course, such as the digestive ones, are found already in solution in the various digestive secretions. Many others, however, are formed and act within the cells. Most of these are readily separated from the cells by suitable solvents. A few have so far proved refractory to extraction, and it was once customary to say that such enzymes are inseparable from the cell structure. In the light of recent successes in extracting some of these, it is probably more accurate to say that a few have so far failed to dissolve in the solvents which have been used. Certainly most of the known enzymes have been removed from the cells in which they occur, and much of our knowledge of enzyme activity has been obtained by the study of cell-free solutions. Waldschmidt-Leitz,³ who has done much of the fundamental work on the protein-splitting enzymes, indeed defined enzymes as "material catalyzers of an organic nature, elaborated by living cells but independent of their presence in operation."

Isolation of Enzymes

Liebig commented on the small amount of emulsin needed to bring about hydrolysis of amygdalin. We know now that his emulsin, as well as Buchner's press-juice from yeast cells, contained relatively enormous amounts of inert substances extracted or pressed out with the active catalytic agents. One of the most important accomplishments in the field of enzyme chemistry has been the separation of the catalysts from the contaminating material in the crude extracts. This has led with some enzymes to isolation of pure crystalline substances, and in all cases to a great reduction in the actual weight of material associated with a given degree of enzyme activity. We shall next consider some of the means used to purify enzymes, partly for the light these procedures throw on the chemical nature of the enzymes themselves.

EXTRACTION FROM CELLS

Except for the few which are secreted in the digestive juices, most enzymes must be extracted from the interior of cells. This involves first a destruction of cell walls. Buchner achieved this by grinding the yeast cells with sand. A similar *mechanical* destruction of cell structure is now achieved by grinding tissue between metal plates in a mincer, or between ground glass surfaces rotated against each other. A second very common

³ Ernst Waldschmidt-Leitz (1894–) was Professor of Biochemistry at the German University of Prague, and later published from the Bayer Institute in Munich. Since 1946 he has been Professor in the University of Erlangen with a private laboratory in Munich. His work has been chiefly concerned with the enzymes of protein hydrolysis and synthesis.

method of rupturing the cell is known as *autolysis* or self-digestion. This depends on the fact that all cells contain enzymes, called *cathepsins*, which are concerned in the living cell with protein synthesis and hydrolysis. In the dead cell the hydrolytic reaction predominates for a variety of reasons. Therefore, if minced tissue is incubated in the presence of an antiseptic to keep it free from bacteria, there results a self-digestion of cell proteins, including the proteins of the cell walls. Some enzymes may, of course, be destroyed in the course of autolysis, but for many others this has proved to be a convenient way of making them accessible to a solvent. Yeast, for example, contains the enzyme sucrase or invertase which catalyzes the hydrolysis of sucrose. The first step in the extraction of this enzyme is autolysis of the cells for several days at room temperature in the presence of toluene to maintain aseptic conditions. There results a complete liquefaction of the cell mass. Spinning in a centrifuge after addition of water throws down some insoluble matter and leaves a cloudy supernatant solution containing the enzyme. A third common preliminary treatment is *dehydration* with acetone or other dehydrating solvent. The finely minced tissue is stirred or allowed to stand with several times its own volume of acetone, and finally filtered. If necessary this process may be repeated several times. After the final filtration, the tissue is dried in air and can then be finely powdered in a mortar. Such acetone-dried preparations often retain their activity for months. Extraction of the powder with a suitable solvent yields a cloudy, crude solution containing the enzyme contaminated with varying amounts of inert soluble material.

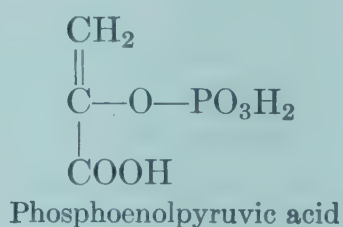
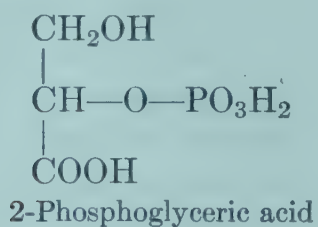
PURIFICATION OF ENZYMES

Given such a crude extract, the next step is separation of the enzyme from as much as possible of the inert material which has dissolved with the catalyst. This requires a method of testing for the presence or absence of the enzyme and usually involves setting up some sort of arbitrary unit in which to express its concentration.

Enzyme Tests. The test for the enzyme will depend upon the type of reaction which it catalyzes. One or two examples should make clear the general principles on which such testing is based. When an ester is hydrolyzed the products are an alcohol and an acid. Of the four substances involved, one, the acid, can be easily identified with a suitable titration indicator. To find out whether or not a given extract contains an enzyme capable of catalyzing the hydrolysis of an ester it is necessary only to add some of the extract to a mixture of the ester and water and to test from time to time to see whether acid is being formed. Another type of testing is used if the enzyme reaction being studied is one which involves the use or evolution of a gas. Delicate and ingenious apparatus is avail-

able for the measurement of small changes in the volume of gas enclosed within the apparatus. Many enzymes catalyze reactions in which molecular oxygen is used. Others either promote the direct evolution of carbon dioxide, or the formation of an acid which, in the presence of bicarbonate, will set free an equivalent amount of carbon dioxide. The presence of the fermentation enzyme can be proved by showing that the solution being examined does, under certain definite conditions, bring about the evolution of carbon dioxide from a sugar solution. With other enzymes a decrease in the volume of gas within the apparatus indicates that oxygen is being used under the catalytic influence of an oxidizing enzyme.

A different sort of test which is simple, elegant, and rapid can be applied to reactions in which the light absorption of the substrate differs from that of its transformation product. There is, for example, an enzyme which catalyzes the dehydration of 2-phosphoglyceric acid to phosphoenolpyruvic acid.



The phosphoenolpyruvic acid absorbs ultraviolet light of 240 $m\mu$ wave length; the substrate transmits this wave length. When a tissue extract is added to the substrate, the appearance of the absorption band at 240 $m\mu$ indicates that the product has formed, and therefore that the enzyme is present. Furthermore, the rate at which the band appears is a measure of the concentration of the enzyme solution.

The purification of a crude enzyme extract involves the removal of much inert material. In one preparation, for example, 16.5 g. of material were extracted, but of this only 1.14 g. remained after purification. To express the ratio of active to inert substances in a solution, it is customary to refer to the number of arbitrary activity units per milligram of dry weight. A unit consists of the amount of enzyme which will change a specified amount of substrate under well-defined conditions. Such an arbitrary unit for sucrase was defined in one study of the enzyme as that amount of enzyme which would reduce the rotation of 4.0 g. of cane sugar in 25 ml. of a carefully defined solution, to zero degrees in one minute. As an enzyme preparation is progressively freed of inert matter, the actual weight of substance required to change a given weight of substrate becomes quite fantastically small.

Separation from Inert Matter. The methods in use for the purification of enzymes are essentially those developed for the purification of proteins.

A very general method makes use of protein precipitants to separate active and inactive material. Repeated precipitations and dissolutions yield at each step two fractions, each of which must be tested for the presence of enzyme. With many extracts so high a proportion of the activity will be found in one fraction that the other can be discarded. Electrophoresis has proved a valuable tool in enzyme purification, as has the method of repeated adsorption on, and elution from, various finely divided adsorbents. The latter process has been useful not only in separation of an enzyme from inactive material, but in concentration of enzyme solutions, since the volume of liquid used for elution may be only a fraction of that from which the enzyme was originally adsorbed.

Some of the procedures which have led to enzyme preparations of high potency are long and complicated and arduous. One method began with 18 months' autolysis! On the other hand it may happen that the process is very simple. The enzyme urease may be extracted at room temperature from finely ground jack bean meal with dilute acetone. During overnight filtration at ice-box temperature the enzyme crystallizes. Urease was, in fact, the first enzyme to be obtained in pure crystalline form. This was announced by Sumner⁴ in 1926. He substantiated his claim that the crystals *are* the enzyme, and not simply a carrier of minute amounts of a much more active substance, by comparing activity before and after repeated recrystallizations. His crystals hydrolyze urea 700 to 1400 times as fast as equal weights of the bean meal from which the enzyme was extracted, and for a given sample this activity is neither increased nor decreased by repeated recrystallizations.

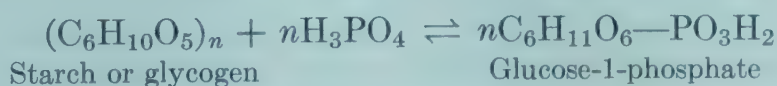
Since 1926 many other enzymes have been prepared in crystalline form, though many more are known to await purification. With some extracts the problem was simply one of ridding a single substance of inert material. In others, what was believed to be a single enzyme proved to consist of a whole congeries of such agents. In these cases the process involved the identification and separation of one new enzyme after another. An outstanding example of this is the so-called "zymase." This is the name which was originally given to the enzyme believed to be present in Buchner's yeast press-juice. Soon it was discovered that in this cell extract there was more than one simple catalytic agent. When the enzyme was dialyzed it lost its activity, but addition of the dialyzate reactivated it. The nonprotein material which passed through the dialyzing membrane was called a *coenzyme*, since it contained small molecules essential

⁴James B. Sumner (1885-) is Professor of Biochemistry at Cornell University, where he has taught since 1914. He prepared the first crystalline enzyme in 1926; shared the Nobel Prize in Chemistry with Stanley and Northrop in 1946. His chief interest has been enzyme chemistry.

to the catalytic activity of an enzyme. The process of separation so begun has gone on until we now know that "zymase" contains at least fourteen enzymes and five coenzymes all involved in the catalytic transformation of glucose to alcohol and carbon dioxide.

Potency of Enzymes. An idea of the phenomenal efficiency of enzymes may be gained from the so-called "turnover number" of a few enzymes. This number expresses the number of moles of substrate transformed per mole of enzyme per minute at a given temperature. It has been estimated for catalase, which catalyzes the breakdown of hydrogen peroxide to water and oxygen, that 1 molecule of it brings about the decomposition of 2.5×10^6 molecules of the peroxide per minute at 0°C . Other enzymes are not quite so spectacular as this, but are nonetheless formidable. The crystalline enzyme which catalyzes the hydration of fumaric to malic acid has a turnover number at 20°C . of 10^5 , while pyruvic acid is reduced in the presence of the proper enzyme at the rate of 8×10^4 molecules, per molecule of catalyst per minute at 38°C .

Nomenclature. In the early years, names were given to individual enzymes at the pleasure of the discoverer. Emulsin was so named, because it was found in an emulsion. Trypsin probably comes from the Greek word meaning "a rubbing," and was chosen because the enzyme was first obtained by grinding pancreas with glycerol. Pepsin means "to cook" or "digest." With the introduction of a rational system of nomenclature in other chemical fields, a system of naming enzymes has come into use in which the ending *-ase* indicates an enzyme. Originally the ending was used with the substrate name, as in urease or sucrase. More recently it has been used more flexibly, especially with words which indicate the type of reaction involved. This is the case with the names given to groups of enzymes which bring about similar reactions. Those which catalyze the hydrolysis of their substrates are called *hydrolases*. Such are all the digestive enzymes. A similar reaction is the splitting of polysaccharides in which phosphoric acid is involved instead of water.



This reaction is spoken of as phosphorolysis, and the active agents are grouped together as *phosphorylases*. Some individual enzymes are likewise named with a view to indicating the type of reaction they catalyze. This results in such names as *carbonic anhydrase* and *succinic dehydrogenase* for the factors which catalyze, respectively, the decomposition of carbonic acid and the removal of hydrogen from succinic acid. Other class names and most individual names can best be acquired gradually, as the functions of the enzymes themselves are developed.

Properties of Enzymes

CHEMICAL NATURE OF ENZYMES

Of the enzymes which have so far been crystallized, all have proved to be proteins. Some, like urease, are simple proteins. Others are conjugated proteins, with prosthetic groups more or less firmly attached. For light upon the structure of the great body of enzymes which are still known only in impure extracts, we have only indirect evidence. We know that the methods which have proved successful in their purification are the same as those used in isolation of individual proteins. We know that enzyme molecules are too large to pass through a dialyzing sac. Most important of all, we know that the properties of enzymes, some of which will be considered in a later section, are not only consonant with the idea that enzymes are protein in nature, but are best explained on that basis. Occasionally it has been reported in the literature that a given enzyme solution was very active, but gave no protein tests. This does not prove that the enzyme is not a protein, but may simply indicate that the solution is too dilute to respond visibly to protein tests. It is not possible, of course, to state categorically that all enzymes are proteins, but all available evidence makes this seem likely.

ENZYMES AS CATALYSTS

Enzymes have been repeatedly referred to in the preceding paragraphs as "catalysts." It will perhaps be worth while to indicate at this point why, in spite of some misleading appearances, it is generally accepted that their function is catalytic. On no other supposition can their remarkable efficiency in terms of their own weight be satisfactorily explained.

Ostwald⁵ defined a catalyst as "an agent which affects the velocity of a chemical reaction without appearing in the final products of the reaction." This does not, of course, imply that it takes no part in the reaction, but simply that the amount and chemical composition of the catalyst are unchanged at the end. When conditions allow, this can best be proved by recovery of the unchanged catalyst. With enzymes this is impossible, both because of their chemical nature, and because they are used in such minute amounts. Often the only proof of their existence is the effect which they exert, and it is a fact that this effect usually diminishes as the reaction proceeds. This is especially true with highly purified enzymes and would appear to argue against their being considered catalysts, since they seem actually to be destroyed in the course of the reaction. It is possible, however, to explain this apparent destruction in other terms. The inacti-

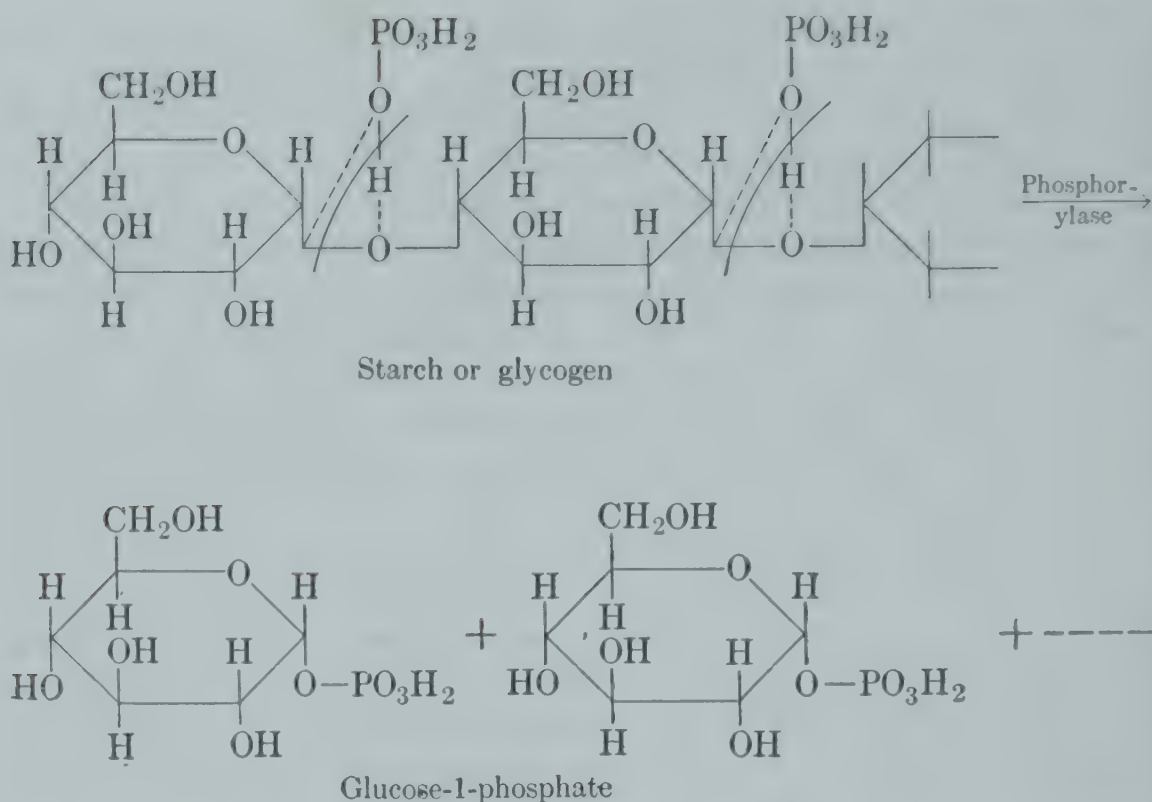
⁵ (Friedrich) Wilhelm Ostwald (1853-1932) was Professor first at the Riga Polytechnicum, and later at Leipzig. He founded the *Zeitschrift für physikalische Chemie* and indeed may almost be said in collaboration with Nernst and van't Hoff to have founded the subject also. He was awarded the Nobel Prize for Chemistry in 1909. After 1906 he worked as a private investigator until his death in 1932.

vation may be brought about by a poisoning or inhibition of the enzyme by one or other of the products of the reaction. For example, the activity of a given amount of sucrase in hydrolyzing sucrose is reduced about one-half if to the mixture of the substrate and enzyme either glucose or fructose is added at the beginning of the reaction. Obviously with this enzyme there would be a similar progressive inhibition as the hydrolysis of the disaccharide resulted in an accumulation of glucose and fructose as reaction products. It must never be forgotten that isolated, purified enzyme systems reproduce very poorly the conditions within a cell. There, in the natural setting, such inhibitory products may be removed through the agency of other enzymes, or they may simply dialyze away as fast as they are formed.

It is generally accepted that a true catalyst does not initiate a reaction which is otherwise incapable of taking place, but simply increases the reaction rate. Here again enzymes seem superficially not to function as catalysts. For example, glucose, normally very stable, acts as substrate for a number of different enzymes, giving rise to different products, none of which appear to form except in the presence of the corresponding enzyme. It can be oxidized enzymatically to gluconic acid or reduced, in the presence of a different enzyme, to sorbitol. The enzymes of yeast transform it into alcohol, while those from one strain of streptococcus degrade it to lactic acid. In explanation of such reactions, J. B. S. Haldane⁶ long ago pointed out that side reactions are the rule in organic chemistry. Even with so stable a substance as glucose, apparently not reacting at all, there may well be an equilibrium mixture in which there are indistinguishable amounts of various reaction products in solution. When there is added to this solution an enzyme which selectively accelerates the rate at which one of the products is formed, it will appear to have "caused" its formation, even though its action is strictly catalytic.

A third distinguishing mark of a typical catalyst is its ability to catalyze both the forward and the back reaction. The classical example is the action of acid upon the rate of saponification or esterification. Whether the starting materials are ester and water or acid and alcohol, the catalysis consists in hastening the rate at which the identical equilibrium is achieved. With many biological reactions, it is true, the equilibrium point is such that the reaction appears to go to completion in one direction. With many enzymes, however, it has been possible to prove that they do catalyze both a forward and a reverse reaction. An interesting example of this is given by the phosphorylases which catalyze the splitting of a polysaccharide by phosphoric acid.

⁶ James Burdon Sanderson Haldane (1892-) was Reader in Biochemistry at Cambridge University, and later held a Professorship at University College, London. Never an experimentalist, he has written voluminously on scientific subjects both for scientists and for the lay reader.



This type of enzyme has been extracted from both plant and animal sources. The plant enzyme acts upon starch and the animal enzyme on glycogen, each yielding the same phosphorylated glucose. If, however, either enzyme is incubated under suitable conditions with glucose-1-phosphate, the reverse reaction takes place and a polysaccharide is formed. Reversibility has been proved for a great number of other enzyme catalyses, even some rather unlikely ones. It seems probable, therefore, that all enzyme actions are theoretically reversible, and that in this respect also, enzymes are true catalysts.

SPECIFICITY OF ENZYMES

One of the most striking characteristics of enzymes is their specificity. In the field of inorganic catalysts some substances, such as finely divided metals, are known to hasten a wide variety of different reactions. Platinum as foil or "platinum black" is such a general catalyst, and some of the metal oxides have also proved to be quite versatile. The enzymes, on the contrary, all require very specific substrates, although their specificities may differ in degree.

Those enzymes which exhibit the least specificity may be said to show *linkage* specificity. Given the presence of the required type of linkage in the substrate molecule, they can act upon it regardless of the character of the radicals held together by that bond. The lipases are the best example of enzymes which exhibit this mild degree of specificity. As their name indicates, they normally catalyze the hydrolysis of simple fats,

splitting the links between long chain fatty acids and glycerol. They will, however, with equal facility split so simple an ester as methylbutyrate, in which are linked an acid and an alcohol, neither of which is found in most fats. Of the known enzymes very few show so little specificity as the lipases. This type of specificity may be indicated by the following equation, in which the use of the indefinite R and R' shows that only the type of linkage is prescribed.



A slightly greater specificity is exhibited by enzymes which have what might be called *positional* specificity. These enzymes attack a given linkage only if it is set in the molecule in proper relation to certain groups or atoms. The phosphatases, for example, belong to the group of enzymes which are known as esterases—a group which also includes the lipases. But the phosphatases hydrolyze only those esters in which the acid involved is phosphoric acid. Such compounds as phosphoglycerol or the hexose phosphates, both of which are true esters, are hydrolyzed by phosphatase, with the freeing of inorganic phosphate. In the equation which symbolizes this degree of specificity, only part of the substrate molecule can be indicated by the general symbol, R. The acid part must be phosphoric acid.



A very pretty example of this type of positional specificity has been afforded by recent work on the protein-splitting enzymes. Pepsin, for example, has long been known to catalyze the hydrolysis of large molecules of the food proteins, breaking peptide linkages and giving rise to smaller fragments. It has now been shown that pepsin attacks a peptide link most readily if the link unites one of the carboxyl groups of a dicarboxylic, and the amino group of an aromatic amino acid, and if certain other conditions are fulfilled. The second carboxyl group of the dicarboxylic acid must be free, nor may its acidity be weakened by the presence of a neighboring free amino group. This is a positional specificity with a vengeance!

A third, and still more exacting type of specificity is *individual* or *absolute* specificity. Enzymes are known which catalyze one specific reaction of one individual substance. Urease, for example, brings about the rapid hydrolysis of urea. However, it is not safe to conclude that an enzyme which is named from an individual substrate necessarily shows this degree of specificity. Sucrase, for example, will split sucrose and various other fructosides as well. It exhibits positional specificity, demanding a glycoside linkage from fructose to some alcoholic hydroxyl group. The substance known as maltase is also rather equivocally named. If it is extracted

from yeast or from the digestive tract it is merely an α -glucosidase, while the maltase from malt is properly named and will attack only maltose. Below are listed a few of the enzymes which exhibit absolute specificity.

	REACTION CATALYZED
Carbonic anhydrase	$\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$
Catalase	$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
Maltase (from barley)	hydrolysis of maltose
Arginase	hydrolysis of arginine
Succinic dehydrogenase	oxidation of succinic acid

A fourth and final degree of specificity may co-exist with either positional or absolute specificity, when the enzyme in question further exhibits toward its substrate a *stereochemical* specificity. This was foreshadowed in various metabolic experiments with tissues and with bacteria. If L-glucose is injected into a rat, 85 per cent of this unnatural sugar is excreted within twenty-four hours, showing that the rat can make no use of the L-form. Bacteria which need glucose in the nutrient medium fail to grow if L-glucose is used instead of the D-form. Brain slices, which take up oxygen regularly if supplied with D-glucose, are unable to oxidize the L-isomer at all. When we turn from experiments of this sort, in which many unknown enzyme systems are involved, to work with pure, or highly purified, enzymes, we still find that many enzymes are built so that they can use as substrate only one particular stereochemical isomer. The use of maltase and emulsin to distinguish between an α - and a β -glucoside was a familiar procedure in the early days of sugar chemistry. The digestive protein-splitting enzymes also exhibit stereochemical specificity; they will act only upon the peptide bonds between α -amino acids of the natural or L-series. In these examples, stereochemical specificity is added to positional specificity. In others, it further defines an already absolute specificity. Arginase hydrolyzes only L-arginine, and lactic dehydrogenase from muscle can oxidize lactic acid only if it has the L-configuration.

Of recent years a rather strange variety of *dual* specificity has been noted with certain enzymes. The first case was that of xanthine oxidase. As its name indicates, this enzyme, which is found in milk and in some animal tissues, catalyzes the oxidation to uric acid of xanthine or hypoxanthine, but not of other purines. This indicates a specificity nearly absolute. But this same substance in highly purified form exhibits a linkage specificity of a much broader sort. It catalyzes the oxidation of a number of aldehydes to the corresponding acids. More recently a highly purified L-amino acid oxidase has shown this same dual effect. It is specific for (a) oxidation of thirteen different amino acids of the L-series, and (b) oxidation of several alpha hydroxy acids, also of the L-series. This

enzyme has been crystallized and in the course of its purification any increase in activity toward one type of substrate was exactly paralleled by a corresponding increase in its activity toward the other. It seems clear that we have a single enzyme exhibiting a more or less rigorous specificity toward two different substrates.

KINETICS OF ENZYME ACTION

Much of our present information about the nature of enzymes has been deduced from a study of the rates of enzymic reactions, and of the factors

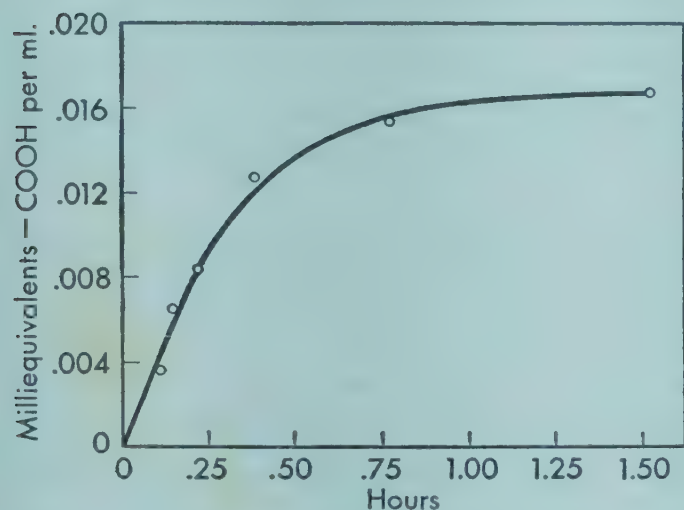


Figure 7.1. The rate of hydrolysis of a 5 per cent casein solution by crystalline trypsin. The increasing concentration of free amino acid was measured by the formol titration. (From J. H. Northrop, *J. Gen. Physiol.*, 16:339, 1932-33.)

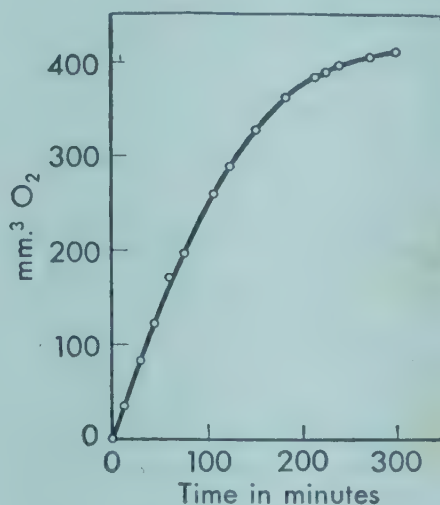


Figure 7.2. Time-activity curve of glycine oxidase. The reaction rate is plotted in terms of the volume of oxygen used. Note that with this enzyme the rate is linear for more than an hour. (From S. Ratner, V. Nocito, and D. E. Green, *J.B.C.*, 152:119, 1944.)

which influence those rates. Theoretically, reaction rate may be measured either (a) in terms of the amount of change occurring in unit time, or (b) in terms of the length of time required to bring about some fixed amount of chemical change. The actual experiments, however, are nearly always carried out by following the change at fixed time intervals, i.e., working in accordance with plan (a). If the amount of chemical change measured is then plotted against time, it is of course possible to read from the curve the time required for any fixed amount of change to take place.

The way in which the experiment is set up, as well as the method used to measure the change, depends upon the nature of the catalyzed reaction. In some experiments, enzyme is added to a rather large amount of substrate and from time to time small aliquot portions are removed to determine how far the reaction has progressed. Other reactions are best followed by setting up a number of small separate flasks and allowing the reaction to proceed for a different length of time in each. If a gas is used

or evolved in the course of the enzymic reaction, the experiment may be carried out in an apparatus designed so that changes in gas volume may be measured directly from time to time. Figures 7.1 and 7.2 show typical enzyme curves obtained by measuring reaction rate in two different ways. The reaction represented in Figure 7.1 is hydrolysis of casein, catalyzed by crystalline trypsin. Since hydrolysis of a protein ruptures peptide bonds and sets free both carboxyl and amino groups, the extent of the reaction could be measured by determining from time to time the increase in the number of either of these groups. For the curve reproduced, aliquot portions of the digest were submitted to a formol titration, and the number of milliequivalents of new carboxyl groups per ml. of solution thus determined. The second curve expresses the rate of oxidation of glycine in terms of the cubic millimeters (microliters, μ l.) of oxygen used by a definite weight of glycine in presence of its specific oxidizing enzyme.

Influence of Activity. It will be noted that the two time-activity curves are of the same general type. They indicate that for only short periods, just after an enzyme has been added to its substrate, is the reaction rate constant, or linear. After that it falls off more or less rapidly until finally the enzyme is completely inactivated. There is probably no single reason for this. Some enzymes are extremely labile, and others maintain a linear rate for quite long periods. This suggests that different factors are involved with different enzymes. It has already been noted that some enzymes may be inhibited by a product of their own activity (see p. 243). In the next few sections we shall consider some factors other than activity which influence the rate of all enzymic reactions. These must be taken into account in any explanation of the shape of the time-activity curves. It is because of the shape of this type of curve that in many experiments with enzymes only the initial, maximum rates are considered.

Influence of pH. Enzymes are all sensitive to changes in the acidity of their environment. Furthermore, most enzymes exert their maximum catalytic effect over a rather narrow pH range, and lose activity rapidly on either side of this optimum. Figure 7.3 shows the effect of a changing pH on the activity of two oxidases in terms of the oxygen uptake in the first twenty minutes. For the glucose oxidase the optimum pH is 5.6; it is relatively inactive at pH 3 and completely so above pH 8. The glycine oxidase, on the other hand, is completely inactive below pH 6, surprisingly inactive at pH 7, and has an optimum at about 8.3. Many enzymes have pH optima close to neutrality; on the other hand pepsin acts at a strongly acid pH while some of the phosphatases, which split phosphoric acid from its esters, require a pH as high as 9 or 10. To achieve and maintain the desired pH, enzyme experiments are always conducted in a buffered medium. The optimal pH values for a few enzymes are indicated in Table 7-I.

This sensitivity to pH changes accords well with our belief that enzymes are proteins. We have seen that the properties of a protein are also dependent upon pH. On the alkaline side of the isoelectric point, proteins

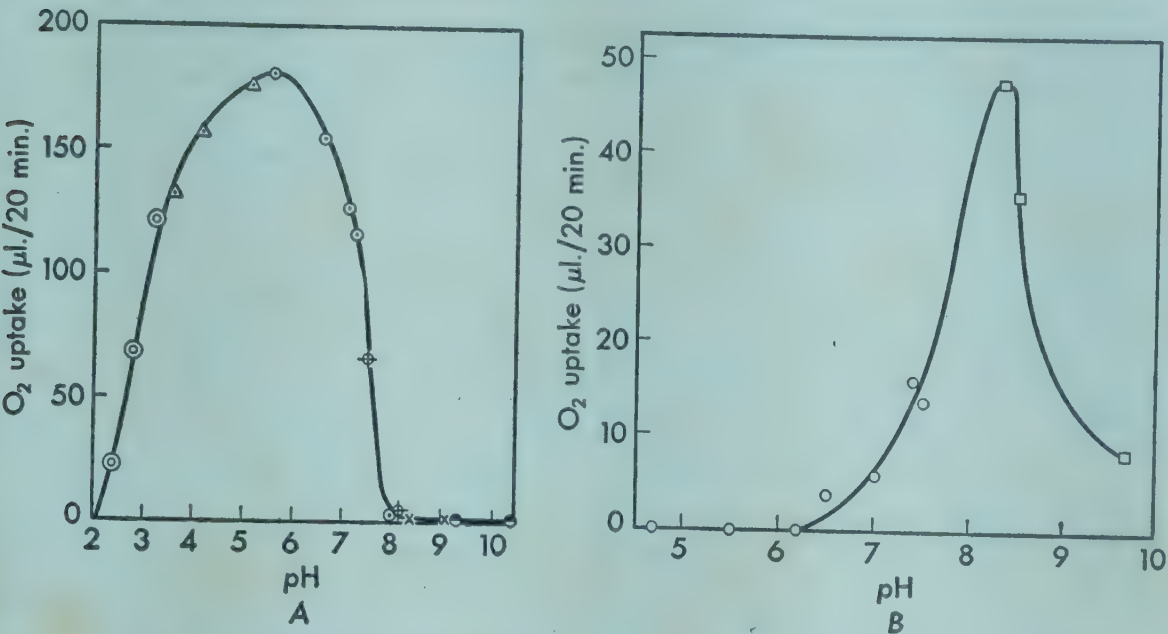


Figure 7.3. The relation between pH and enzyme activity. Curve A. The effect of pH on the activity of glucose oxidase, reported in terms of the oxygen uptake in the first 20 minutes. The different symbols marking the points on the curves indicate the use of several different buffers. (From D. Keilin and E. F. Hartree, *B.J.*, 42:221, 1948.) Curve B. The rate of oxygen uptake in the presence of glycine oxidase as a function of pH. (From S. Ratner, V. Nocito, and D. E. Green, *J.B.C.*, 152:119, 1944.)

exist as anions and react with metallic or other cations, but they react with such an anion as the bromide ion only on the acid side. At the isoelectric point various physical properties such as swelling, solubility, and conductivity are at a minimum, and rise rapidly on either side of this pH. This has been interpreted to mean that the molecular architecture of the ampholyte, the shape of its surface and the charges upon it, depend upon the acidity of the medium. If, then, enzymes are proteins they would

TABLE 7-I. OPTIMAL pH OF A FEW REPRESENTATIVE ENZYMES

Enzyme	Substrate	pH
Pepsin	Native proteins	1.5-2.2
α-Glucosidase	α-Methylglucoside	5.4
Maltase	Maltose	7.0
Lactase	Lactose	5.7
"Zymase"	Glucose	6.2
Amylase	Starch	6.2
Urease	Urea	6.6
Catalase	Hydrogen peroxide	7.0
Trypsin	Proteins	7.8
D-Amino acid oxidase	D-Amino acids	9.0
β-Glycerophosphatase	β-Glycerophosphate	9.5-9.9

be expected to share these properties and in particular to show special characteristics at a pH corresponding to their isoelectric points.

Influence of Temperature. The influence of increased temperature upon an enzymic reaction is twofold. Other things being equal, increasing the temperature increases the rate at which any chemical reaction takes place. But if the reaction is catalyzed by an enzyme, other things are far from

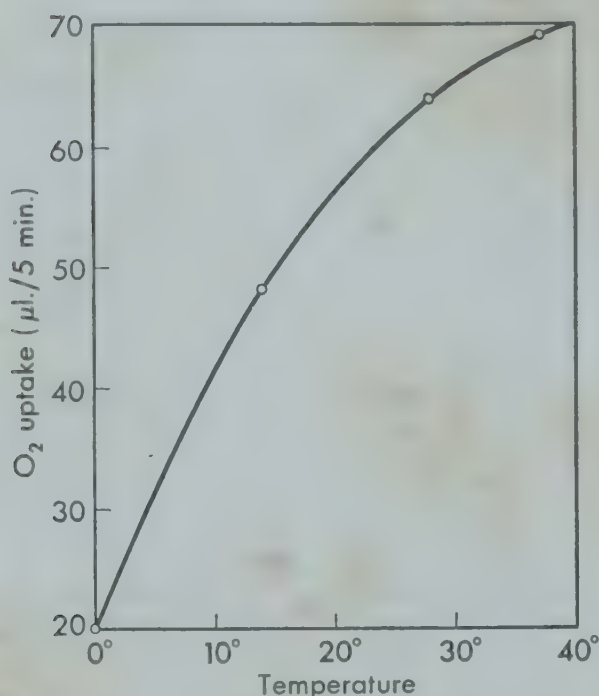


Figure 7.4. Effect of temperature on the activity of glucose oxidase. Note that the temperature coefficient of the enzyme activity falls off with rising temperature, which means that temperature inactivation of the enzyme accelerates as the temperature rises. (From D. Keilin and E. F. Hartree, *B.J.*, 42:221, 1948.)

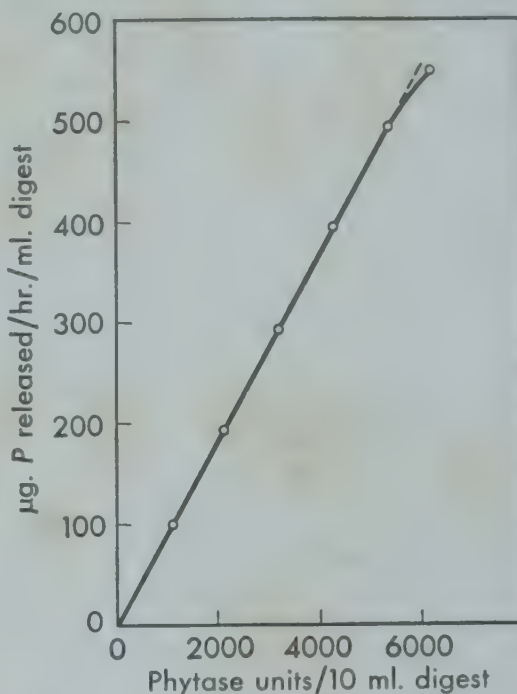


Figure 7.5. Relation between enzyme concentration and activity in the presence of a purified phytase from wheat. The enzyme hydrolyzes phytic acid (inositol hexaphosphoric acid) and sets free inorganic phosphate. Note that the rate is linear over very nearly the entire concentration range which is plotted. (From F. G. Peers, *B.J.*, 53:104, 1953.)

equal. Most enzymes function *in vivo* at moderate temperatures, and are inactivated to a greater or less degree when the temperature is raised. All enzymes are destroyed by boiling their solutions. The extent of the inactivation of a given enzyme depends upon the temperature and upon the time during which the enzyme is exposed to its influence. For this reason the optimum temperature for an enzyme cannot be stated definitely and unconditionally, as if it were a fixed property of that enzyme. If the activity of an enzyme is to be used over a brief period, it may well be that the increase in reaction rate at a higher temperature will more than compensate for the gradual inactivation of the catalyst. If, however, the reaction must be of longer duration, it is probable that a greater amount of substrate will ultimately be acted upon if the temperature is not high enough to inactivate the enzyme rapidly. In general, enzymes

from the tissues of warm-blooded animals act best at about 37°C., while plant enzymes have an optimum temperature in the neighborhood of 25°C. Figure 7.4 illustrates the effect of temperature on an oxidizing enzyme from the mold *Penicillium notatum*.

This property of heat sensitivity is another which indicates that enzymes are proteins. The temperature coefficient of a reaction is defined as the ratio of the rate at $(T + 10)^\circ$ to the rate at T° . When we say that for most chemical reactions the temperature coefficient is 2, we mean that for each 10° rise in temperature the reaction rate is doubled. There are two conspicuous exceptions to this. The temperature coefficient for heat inactivation of enzymes is enormously greater than 2, and may be of the order of several hundreds in the neighborhood of 70°C. The only other known reaction which has a temperature coefficient of this order of magnitude is the denaturation of proteins. The nature of thermal denaturation is still a matter for argument, but it is known that denaturation at slightly elevated temperatures is reversible. This is true also of heat inactivation of many enzymes, if the temperature used is not extreme and is not too long continued. Protein denaturation and enzyme inactivation both become irreversible at higher temperatures. This parallelism is interpreted as evidence that the active catalyst is a native protein.

Influence of Enzyme Concentration. The rate of an enzyme reaction, when pH and temperature are fixed and when excess of substrate is available, is directly proportional to the enzyme concentration. Figure 7.5 illustrates this relationship in the hydrolysis of phytic acid by a phytase derived from wheat. In this experiment the initial rate is linear over a wide range of enzyme concentrations.

Influence of Substrate Concentration. In a series of experiments in which the pH, temperature, and enzyme concentration are all held constant, the rate of the initial reaction will depend upon the substrate concentration, up to a definite limiting value. Beyond this, any further increase in substrate concentration brings about no further increase in reaction rate. This is shown in Figure 7.6, which shows graphically how the rate of oxidation of glycine varies with the concentration of the substrate. Substrate concentration is plotted against the initial reaction rate measured in terms of the volume of oxygen consumed in a fixed time interval. The flattened curve indicates that with the amount of enzyme used, about 0.5 molar glycine reacts at a maximum rate.

Where a reaction involves two substances, we should perhaps speak of two substrates. This is true, of course, in any hydrolysis. But since the concentration of water is very nearly constant in any reaction mixture using dilute solutions, we seldom think of water as a possible substrate, and in any case cannot study the effect of changes in its concentration. But in reactions in which a substrate is oxidized by molecular oxygen

there are clearly two reactants, and the concentration of either may be varied. In such reactions the rate varies directly with the concentration of either substrate. In Figure 7.7 are shown the data from an experiment with glucose oxidase, in which the oxygen tension was varied while the glucose concentration was constant. The curve is of the same general type as the previous one, showing an enzyme activity in pure oxygen nearly $2\frac{1}{2}$ times that in air. We shall see in a later section how this dependence

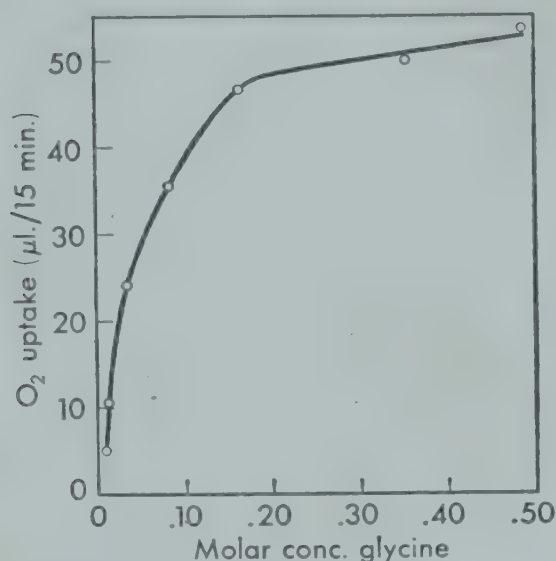


Figure 7.6. Rate of oxygen uptake in the presence of glycine oxidase as a function of the concentration of the substrate, glycine. (From S. Ratner, V. Nocito, and D. E. Green, *J.B.C.*, 152:119, 1944.)

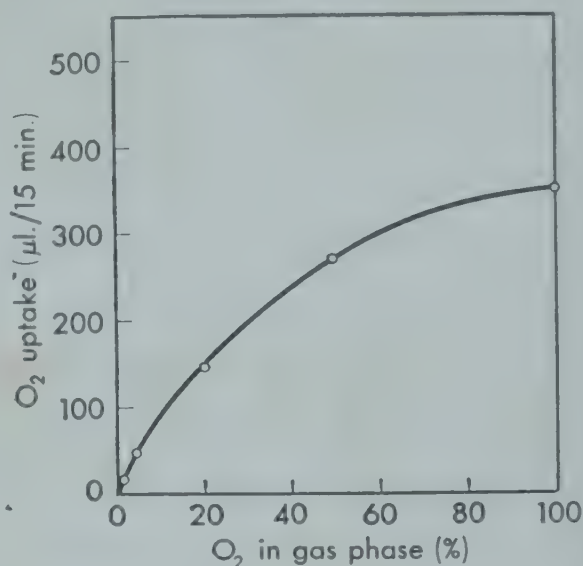


Figure 7.7. Effect of oxygen tension on the activity of glucose oxidase. (From D. Keilin and E. F. Hartree, *B.J.*, 42:221, 1944.)

of enzyme rate upon enzyme concentration and upon substrate concentration can be explained.

ACTIVATION OF ENZYMES

It frequently happens that in the course of purification an enzyme is separated from some substance, present in the original tissue and essential to its activity. These essential substances or "activators" are of various types and function in one of two ways. In some cases they actually transform an inactive protein into an active enzyme; in others they themselves coöperate in one way or another in the enzymic process.

Pro-enzymes. It has long been known that certain digestive enzymes, especially the proteolytic ones, are secreted in an inactive form. Pepsin, for example, is secreted by special cells in the gastric mucosa and was first identified as a proteolytic enzyme in the gastric juice. However, attempts to obtain the same enzyme by extraction of the secreting mucosa itself yielded only an inactive precursor of pepsin. Such inactive molecules are known as *pro-enzymes* or *zymogens*, and their nature is indi-

cated in their names by the use of the prefix *pro-* or the suffix *-ogen*. Pepsinogen is the substance which will become pepsin on activation; prorennin is the precursor of rennin.

Pepsin differs from pepsinogen in elementary analysis, in molecular weight, and in optical rotation. With other pro-enzymes the differences are smaller and manifest themselves in differences in crystalline form and in solubility. It is believed that the transformation of a zymogen consists in actual changes, great or small, in the structure of the protein molecule. With some zymogens this may be brought about by a simple change in hydrogen ion concentration. Pepsinogen is activated as soon as the *pH* of its solution drops below 6. Or the activator may be itself an enzyme. Enterokinase is the name given to an enzyme which activates trypsinogen; the trypsin so formed then serves not only as a proteolytic enzyme but to activate another digestive pro-enzyme present in the same secretion. This elaborate system of activations makes it certain that proteolytic hydrolysis will not begin by destroying *in situ* the very cells that secrete the necessary enzymes.

Activation by Inorganic Ions. Some enzymes, even though secreted in an active form, still require the collaboration of some nonprotein substance. This type of activation differs from that just outlined in that the activator is believed to take part in the catalytic reaction.

There are a number of enzymic reactions for which the presence of inorganic ions other than hydrogen is essential. Salivary amylase, for example, may be inactivated by removal of small molecules by dialysis. Addition of various inorganic anions causes a reactivation, though chloride ion, which occurs naturally in saliva, is the most effective. Other enzymes have more specific requirements. The activity of kidney phosphatase is increased greatly by very low concentrations of magnesium ion. A number of other enzymes are activated by the divalent cations of manganese, cobalt, or nickel. With some of these it appears that the active enzyme is an easily dissociable compound of protein and activating cation, i.e., a metallic salt of the protein. It has been suggested that a divalent cation may function by holding the enzyme protein with one valence and the substrate momentarily with the other. Quite recently evidence has been adduced that one function of this type of activator may be to induce a preliminary change in the substrate. At present there is no way to decide whether all the activations by inorganic ions make use of the same mechanism, or whether there are as many different mechanisms as activators.

Coenzymes. When a crude enzyme solution is dialyzed there pass into the dialyzing fluid not only inorganic ions, but many small organic molecules. Some of these organic substances have proved to be essential to the activity of the enzymes with which they were extracted. This was

first discovered in work with the yeast enzymes known collectively as "zymase." The unknown essential factor in the dialyzate was then referred to as "cozymase." Since then it has been discovered that many enzymes occur in the tissues as conjugated proteins. The prosthetic groups of most such compounds can be removed, leaving a protein part or *apoenzyme* which is inactive but which can be reactivated by replacing the prosthetic group. When separated from their respective proteins these groups are known as *coenzymes*. The complete, active conjugated protein is sometimes called a *holoenzyme*. The function of the coenzyme is not, as with enterokinase, to induce a change in a pro-enzyme. The coenzyme participates in a quite characteristic way in the catalyzed reaction. The way in which this occurs is described in the following section.

THE OXIDIZING ENZYMES

The enzymes which are concerned with cell oxidations have nearly all been isolated, not as simple proteins, but as conjugated proteins. If the protein of such a complex is separated from its prosthetic group, neither moiety is active. The active enzyme can usually be reconstituted by putting the two parts together again. A later chapter will be devoted to the activities of this group of enzymes; at this point we need only consider their general properties.

A suitable example of the oxidizing enzymes is the D-amino acid oxidase which is found in various animal tissues and catalyzes the oxidation of any D-amino acid. It has been prepared in a highly purified form, and its properties may be considered typical of the oxidizing enzymes.

If D-amino acid oxidase, that is, the complete conjugated protein, is added to alanine, oxygen is absorbed and the following reaction takes place:

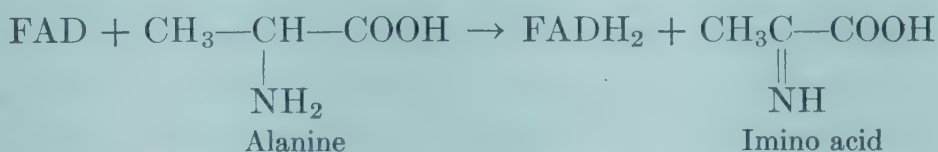


If the solution of the purified enzyme is made slightly alkaline the conjugated protein dissociates into a protein part which is inactive as an enzyme, and a coenzyme which has been identified as a complex flavinadenine dinucleotide, the name of which is commonly abbreviated to FAD. Though neither part alone has any oxidizing action, when they are both added to alanine oxygen is again absorbed. This indicates that the two parts have united to form the complete conjugated enzyme again. If, instead of adding a large amount of dinucleotide, graded small amounts are added to separate, identical samples of the enzyme protein, the activity of each mixture is strictly proportional to the concentration of the dinucleotide. This is shown in Figure 7.8 in which the rate of oxygen uptake is plotted against the dinucleotide concentration. When enough dinucleotide has been added to provide a prosthetic group for each molecule of enzyme

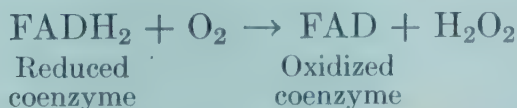
protein, further increases in the concentration of dinucleotide have no further accelerating effect. This is indicated by the plateau in the curve.

The structure of flavinadenine dinucleotide is given in Chapter 14. For present purposes the only property with which we are concerned is a reversible reaction which takes place entirely in the flavin part of the molecule. This part of the dinucleotide is capable of undergoing reversible oxidation-reduction. In the oxidized form the substance is yellow. When it is reduced it adds two hydrogens and the leuco compound so formed is colorless. When the complete oxidase, composed of colorless protein and yellow dinucleotide, is added to alanine, the yellow color is bleached. If the solution is then aerated by vigorous shaking, the color is restored. Since the dinucleotide is the only colored substance present, this indicates that the alanine reduces it to the leuco form, and the oxygen of the air re-oxidizes the leuco form to the original yellow molecule.

Clearly then the reaction indicated above in a single equation takes place in steps, and a transfer of hydrogens from the alanine to the flavin compound is the first of these. This may be indicated schematically:



Since molecular oxygen restores the color of the leuco-FADH₂, it must react, not with the alanine directly, but with the reduced coenzyme.



Since other reduced flavin compounds may be oxidized by molecular oxygen without the intervention of an enzyme, this reaction is probably spontaneous. Likewise, the third step by which pyruvic acid is formed is believed to be nonenzymic.

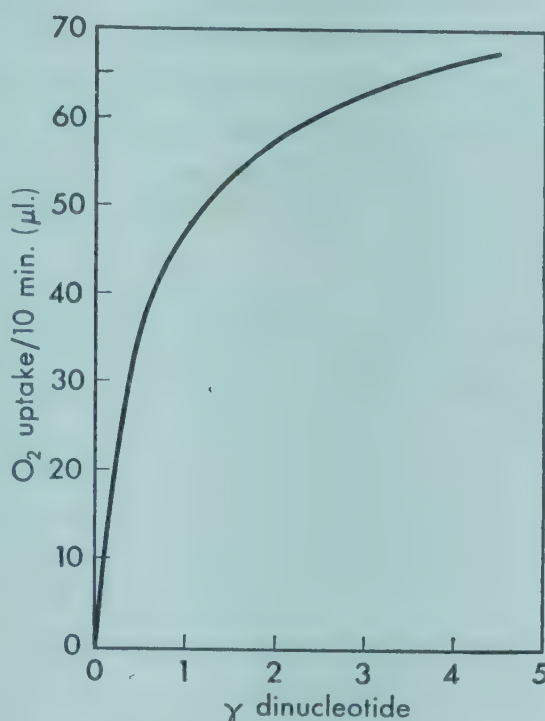
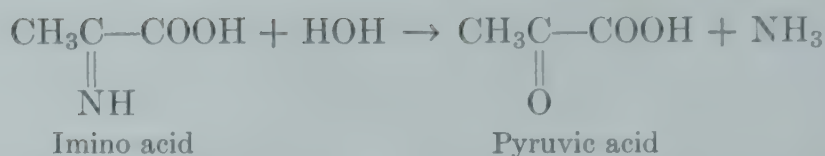


Figure 7.8. Relationship between rate of oxidation by D-amino acid oxidase and concentration of the coenzyme (FAD). The linear portion of the curve indicates that the coenzyme forms a compound with the enzyme protein in a definite stoichiometric ratio. (Data from O. Warburg and W. Christian, *B.Z.*, 298:150, 1938.)



The coenzyme is thus seen to go through a cycle, beginning and ending with the yellow oxidized form. One molecule can therefore bring about the oxidation of a great number of molecules of alanine, by accepting hydrogens from the activated substrate, and passing them on to molecular oxygen.

For the oxidation of alanine both parts of this enzyme are required. The activated substrate cannot hand on its hydrogens to any readily reducible substance which happens to be present. It is not "activated" in general, but activated to give up hydrogen to just the particular dinucleotide which is the coenzyme for its oxidation. Nor should it be assumed, from the fact that the actual reaction involves only the dinucleotide, that the specific protein is unimportant. It is only in the presence of the protein that the hydrogen transfer can take place. If this same flavinadenine dinucleotide is associated with a different enzyme protein, alanine is not oxidized, but D-glucose is. In fact there are known at least ten different enzymes, for which the same dinucleotide acts as coenzyme. The substrate specificity of each must then reside in the protein part of the conjugated molecule.

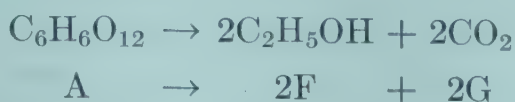
ENZYME INHIBITORS

We have already seen that enzymes are inactivated by even slightly elevated temperatures, and that they are completely destroyed by boiling. There are a number of chemical agents which also completely destroy the activity of enzymes. Some of these are protein precipitants, including such substances as trichloroacetic acid and phosphotungstic acid. They are often used in enzyme studies to stop the reaction at a specified time by precipitating all the proteins.

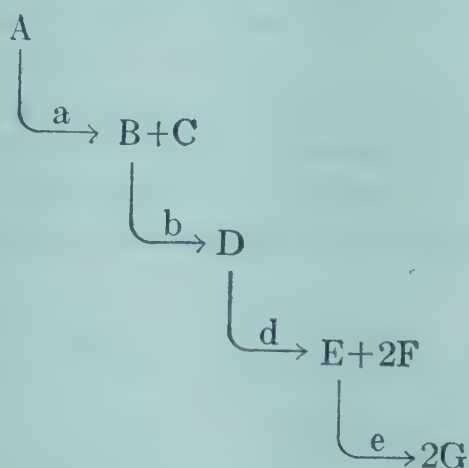
Other enzyme inhibitors or poisons act more selectively. Many respiratory enzymes are inhibited by very low concentrations of cyanide. This is interpreted to mean that a metal, usually iron, forms an essential part of the enzyme or coenzyme. When this metal is blocked by being engaged in a cyanide complex, the enzyme can no longer exert its catalytic function. Other enzymes, to the activity of which a metallic ion is not essential, are unaffected by cyanide. These include most of the hydrolytic enzymes. Many of these, however, are inactivated by oxidizing agents, and can be restored to activity by such a reducing agent as reduced glutathione. Other common inhibitors are sodium fluoride, sodium azide (Na_3N), and moniodoacetate. Any detailed study of the phenomenon of inhibition, and of the conclusions which may be drawn from the sensitivity of an enzyme to specific inhibitors, is beyond the scope of this book.

One very important use of inhibitors, however, can suitably be considered at this point.

It was noted earlier that the fermentation of glucose involves many different enzymes, all of which are present in a yeast press-juice or extract. This means that a series of separate transformations is involved, with a number of different intermediate products, although the over-all reaction may be expressed:



This may be indicated schematically as follows, the capital letters standing for different substances, and the small letters for the corresponding enzymes:



When the tissue extract which contains enzymes a, b, d, and e and their essential coenzymes is added to a solution of A, the reaction may appear to be a simple transformation of A into F and G, but this is not so. To follow the actual course of such a reaction the intermediate substances must be identified. Since B is immediately transformed into D, and D in turn is acted upon by its enzyme to form E, etc., it is impossible under ordinary conditions to isolate any except the end products. But if an enzyme poison is added which selectively inactivates enzyme d, the transformation of substance D will not take place. If enzymes a and b are unaffected by the poison, D may well accumulate in such quantity that it can be separated from the mixture and identified. By such methods many apparently simple enzymic transformations have been shown to consist of a series of interlocking reactions. The unraveling of the complex cycle of reactions involved, for example, in the use of glycogen by muscle was facilitated by the use of selective inhibition.

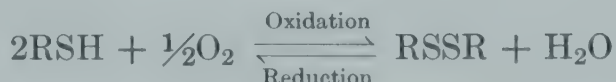
Theory of Enzyme Action

ENZYME-SUBSTRATE COMPOUND

It was Emil Fischer who first suggested that the specificity of enzymes must depend upon each one's bearing to its substrate the relation of a

key to its lock. We have seen that the molecular architecture of the substrate-lock may be rigidly prescribed. For a very few out of all the thousands of enzyme-keys we have a little evidence which indicates that their activity depends upon the presence in their molecules of definite chemical groups. It has been shown, for example, that the activity of certain enzymes depends upon the presence of unsubstituted tyrosine residues in the enzyme protein. If the tyrosine is acetylated or iodinated a decrease in activity results. This has been shown to be true for pepsin, for invertase, for chymotrypsin, and for a number of other enzymes. Pancreatic amylase requires free primary amino groups in the enzyme, but does not depend upon the intact tyrosine grouping. Some of the alkaline phosphatases, on the other hand, are fully active only if there are present both free amino groups and unsubstituted phenolic hydroxyl groups.

There is a large and growing body of evidence that for a wide variety of different enzymes, the presence of intact sulfhydryl groups is essential. We have already seen in connection with glutathione that this is a labile group which can be reversibly oxidized and reduced, the oxidation product being the disulfide form.



If certain enzymes are oxidized, it is found that they are partially inactivated and that the residual activity is proportional to the number of free sulfhydryl groups remaining. If a reducing agent is added to the inactivated enzyme, the activity returns as the sulfhydryl groups are regenerated. The enzymes which have been shown to require free sulfhydryl groups now comprise a formidable list, including esterases, proteinases, dehydrogenases, and oxidases.

All theories of enzyme activity have assumed some sort of union between enzyme and substrate. At one time there was much discussion as to whether this union was true chemical combination, or simply a physical adsorption. The line between the two is no longer so sharply drawn, and the type of union may vary from enzyme to enzyme. Especially with such unspecific enzymes as the lipases, the enzyme-substrate complex may possibly be formed by a process which approximates physical adsorption though this is hard to define exactly. Where the specificity is extreme it is difficult to avoid the conclusion that a reaction in the chemical sense takes place between an essential group or groups in the enzyme and some part of the reaction center in the substrate. The growing body of evidence showing that the activity of enzymes depends upon the presence of specific atomic groupings, of course adds weight to this theory. Even though in the majority of enzymes such groups have not been identified, it is clear that they must exist, and for the purpose of binding the substrate to the

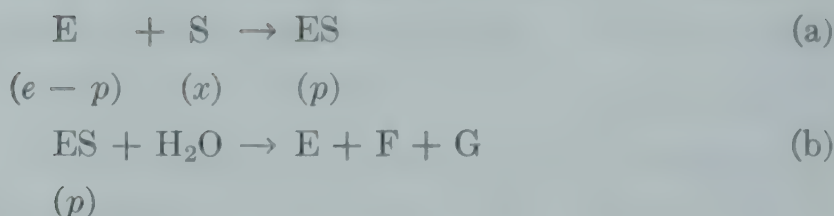
enzyme protein. The actual linkages may be of different kinds, involving strong bonds which are electrostatic in nature, or covalent bonds, or hydrogen bridges.

Competitive Inhibition. The facts of competitive inhibition early offered indirect evidence for the formation of enzyme-substrate complexes. Some enzyme poisons inhibit enzyme action whether the concentration of substrate is high or low. But others bring about a conspicuous degree of inhibition only in the presence of a low substrate concentration. With these enzymes the percentage depression of activity by the inhibitor decreases regularly as the amount of substrate is increased. Such inhibition is spoken of as competitive. Malonate, for example, inhibits in this way the dehydrogenation of succinate by succinic dehydrogenase. This is explained by the close structural relationship between succinic and malonic acids. If the concentration of the normal substrate, succinic acid, is low, malonate, by virtue of its similar structure, is able to form a compound with the active center of the enzyme, thus blocking the formation of an enzyme-succinate complex. Presumably the inhibitor-enzyme compound is stable, and the enzyme involved is thus effectively rendered inactive. But if there is present a large excess of succinate, it is able successfully to compete with the malonate and to occupy a high proportion of the active enzyme centers. Under these circumstances the inhibition caused by a given amount of malonate is relatively small. Other examples of competitive inhibition are known, and all strengthen our belief in the existence in the enzyme of a specific active center or centers which function by forming a compound with the substrate.

The Michaelis ⁷ Constant. One of the strongest arguments for the formation of an enzyme-substrate complex depends upon a mathematical argument put forward by Michaelis forty years ago. He assumed that such a compound does form and then worked out an equation relating the velocity of enzyme action to substrate concentration. Given such an equation, it is a common procedure to compare the relationships which it expresses with those same relationships experimentally determined. If the equation based on an assumption proves to be in agreement with the experimental results, our belief in the original assumption is correspondingly strengthened.

If E stands for a hydrolytic enzyme, S for its substrate, and F and G for the products of an enzymic hydrolysis, the complete reaction would involve the following two steps, assuming that an enzyme-substrate compound is an obligatory intermediate:

⁷ Leonor Michaelis (1875–1949) was born in Germany and practiced medicine there until 1922. He was Professor of Medicine at Nagoya University, 1922–1926; member of the Rockefeller Institute for Medical Research in New York from 1929 until he became Emeritus in 1941. He is chiefly known for the application of physical chemistry to biology and medicine.



Now let e stand for the molar concentration of the enzyme, x for that of the substrate, and p for that of the ES compound. Assuming that the molar concentration of the substrate is, as is usually the case, very much larger than that of the enzyme, the formation of the compound would not appreciably decrease the concentration of the substrate. It would, however, have a significant effect on the concentration of the enzyme. For this reason, the equilibrium concentration of the enzyme is $e - p$, but that of the substrate may still be considered equal to x .

Applying the Law of Mass Action to reaction (a), and allowing K_m to stand for the dissociation constant of the ES compound, we have:

$$K_m = \frac{x(e - p)}{p} \quad \text{or} \quad p = \frac{xe}{K_m + x} \quad (1)$$

K_m is known as the Michaelis constant. In actual practice it is seldom possible to evaluate either e or p , and these terms must therefore be eliminated from the equation before we can compare it with experimental results.

The rate at which the ES compound decomposes will depend on its concentration in the solution, and on the concentration of the other reactant, water. Since the concentration of water under these conditions is approximately constant, we may write

$$v = kp \quad (2)$$

where v is the velocity of reaction (b) and k its velocity constant. Substituting in this equation the value of p from equation (1) we have:

$$v = \frac{k ex}{K_m + x} \quad (3)$$

This still includes the term expressing the enzyme concentration, and so requires further modification.

Obviously if the concentration of the substrate is large enough, all of the enzyme will be in the ES compound, and e will be equal to p . The reaction velocity under these conditions will be at a maximum for a given amount of enzyme, since all of it is saturated with substrate. If V stands for this maximum velocity,

$$V = ke \quad (4)$$

when the substrate is in large excess. Substituting V for ke in equation (3), we have:

$$v = \frac{Vx}{K_m + x} \quad (5)$$

as an expression of the velocity of the reaction in terms of (a) the maximum velocity possible for a given amount of enzyme, (b) the concentration of substrate, and (c) the dissociation constant for the ES compound. How well does this theoretical equation agree with the facts?

The Michaelis equation (equation 5) may be transformed to read:^s

$$(K_m + x)(v - V) = -K_m V$$

Since K_m and V are both constants for any fixed amount of enzyme, this equation becomes $(x + k_1)(y - k_2) = -k_1 k_2 = -K$, in which x stands for substrate concentration and y is reaction velocity. The theoretical curve for such an equation is plotted in Figure 7.9 and is seen to be a rectangular hyperbola. But we have already considered some experimental data relating the rate of reaction (v) to substrate concentration (x). This was plotted in Figure 7.6, which is clearly a curve of the same general type as the theoretical one.

Other assumptions made by Michaelis are borne out by the experimental facts. According to his equations, the reaction velocity in the presence of a large excess of substrate depends only upon the enzyme concentration ($V = ke$). This we have found to be true (Figure 7.5). Fur-

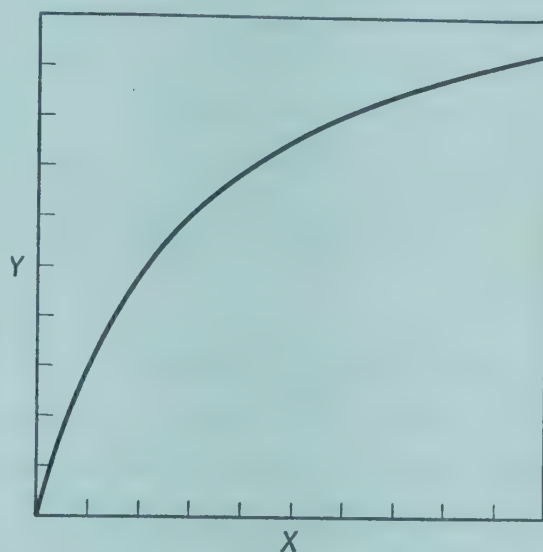


Figure 7.9. Theoretical curve for the equation $(x + k_1)(y - k_2) = K$. The values were obtained by assigning arbitrary values to k_1 and k_2 and then calculating the values of y which corresponded with a series of possible values of x .

thermore, if a constant amount of enzyme is considered, all the factors on the right of equation (3) are constants except x , which represents substrate concentration. In other words, the velocity of the reaction in the presence of a fixed amount of enzyme is dependent only upon the substrate concen-

^s (1) $vK_m + vx = Vx$

(2) $vK_m + x(v - V) = 0$

Subtract $K_m V$ from both sides

(3) $K_m(v - V) + x(v - V) = -K_m V$

(4) $(K_m + x)(v - V) = -K_m V$

tration, so long as that is the limiting value, so long, in other words, as the concentration of the ES compound (p) is less than that of the enzyme (e). This also fits the experimental facts. In Figure 7.6 it is apparent that the velocity of the initial reaction is proportional to the substrate concentration only up to a definite limiting value. It seems reasonable to conclude that the limiting value is that concentration of substrate at which all of the enzyme has been engaged in the ES compound.

The curves shown in Figures 7.5 and 7.6 were chosen because the relationships they indicate have been found to be typical for many uncomplicated enzyme reactions. When we find that they are so well explained in terms of the Michaelis theory and equations, our belief in the reality of the ES compound is correspondingly strengthened.

As a matter of fact the existence of such a compound, so long debated, is no longer in doubt. The first direct proof of its existence was obtained in 1943 in experiments with the oxidizing enzyme, peroxidase. When this enzyme activates hydrogen peroxide it very rapidly forms a compound which can be recognized by the appearance of a new absorption band. Since that time other such enzyme-substrate compounds have been identified, and it is now generally accepted that a combination of enzyme and substrate is the first step in biocatalysis. If in spite of this, the Michaelis derivation has been included here it is because the Michaelis constant is a fundamental one in enzyme kinetics and is therefore important in its own right.

MECHANISM OF CATALYSIS

Assuming then that an enzyme does form a compound of unspecified type with its substrate, various suggestions have been put forward as to the mechanism of the catalysis. Many years ago it was suggested that for formation of the compound, an enzyme must be able to unite with two different atomic groups in the substrate. Recent work on enzyme specificity certainly suggests that there must be at least two such points of contact. The diagram shown in Figure 7.10, taken from a paper by Pigman⁹ illustrates graphically the way in which hydrolysis of a glucoside might progress if its enzyme acted by attaching itself to the substrate at two points. Area *I* represents that part of the enzyme in which its greatest specificity resides; it combines only with D-glucose. Area *II* is less specific and will consequently unite with any one of a number of different groups, provided they are attached through an α -glucosidic link to D-glucose. In the first stage of the reaction each area binds its specific group to form the

⁹ W. Ward Pigman (1910-) is now Associate Professor of Biochemistry at the University of Alabama. The book *Chemistry of the Carbohydrates* which he wrote in collaboration with the late R. M. Goepf is the standard modern work on that subject.

complex. This complex then adds a water molecule, which immediately reacts to rupture the glucosidic linkage. In the final stage the cleavage products leave the surface of the enzyme, which is then ready to activate another substrate molecule.

The nature of this activation is now, as in the time of Liebig and Pasteur, a complete mystery, though attempts to explain it have kept pace with

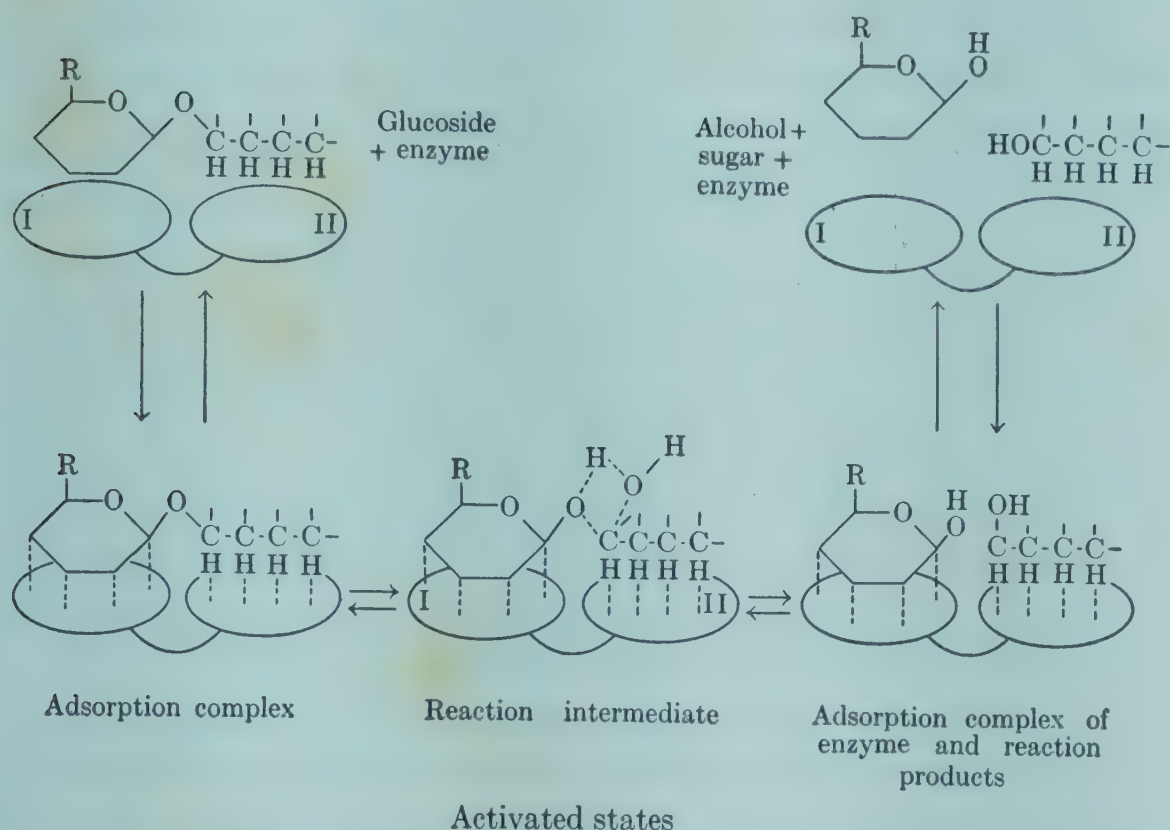


Figure 7.10. Diagrammatic representation of the enzymic hydrolysis of a glucoside. The two oval areas represent active regions on the surface of the enzyme, area I being extremely specific and area II less so. (From W. W. Pigman in *Adv. in Enzym.*, IV:43. Copyright 1944, Interscience Publishers, Inc., New York-London.)

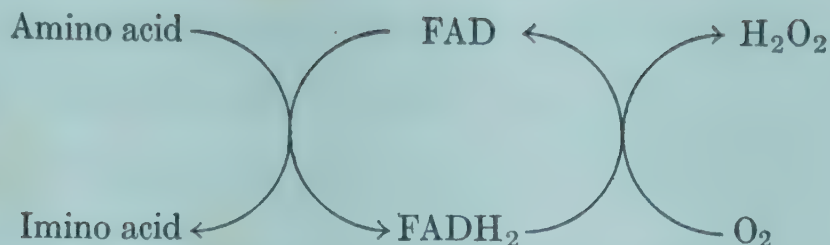
our expanding knowledge of the nature of atoms and molecules. It has been suggested that the substrate may, in the complex, be held rigidly in a position which is advantageous for the addition of water at the glucosidic linkage. It may be, of course, that the water also forms a compound with the same enzyme protein. In this triple complex, water and glucoside may be brought into, and held for an appreciable time in, a special juxtaposition not only with each other but with the surface forces of the enzyme protein. One further suggestion seems worth quoting, for comparison with Liebig's theory of one hundred years ago. In 1844 Liebig wrote: "Sugar breaks down to alcohol and carbon dioxide as a result of a disturbance of the equilibrium in the attraction of its elements caused by a substance whose elements are in a state of motion." Pigman writing in 1944 gave one possible version of the nature of enzymic catalysis in these

words: "During the period of combination of enzyme and substrate, which probably is very short, the translational and vibrational motions of the substrate molecule are restricted and the corresponding energies may provide one source of the activation. . . ." It has happened often in the history of science that a man of genius has made an inspired guess which could be authenticated only after the lapse of years. In Liebig's case, the theory seems to have come full circle!

The speculations in the preceding paragraphs are concerned with those enzymes which are simple proteins. The functional parts of the protein molecules which are referred to as areas *I* and *II* have, for the most part, not been characterized chemically. Presumably the sulfhydryl, phenolic, and other groups which have been found to be essential for the activity of certain enzymes, constitute such areas in those catalysts.

Mechanism of Enzymic Oxidation. The oxidative reactions which take place in cells involve at least three different substances, the enzyme protein, a coenzyme, and a substrate molecule. It has recently been proved, and has long been assumed, that the catalysis must involve not only a union of enzyme protein (apoenzyme) and substrate but a formation of some sort of complex between apoenzyme and coenzyme. The course of oxidation of alanine by molecular oxygen in the presence of D-amino acid oxidase may again be taken as a typical example of an enzymic oxidation, and the series of reactions outlined as follows:

1. On the surface of the specific protein a compound is formed between protein and coenzyme, this constituting the complete oxidizing enzyme (holoenzyme).
2. The complete enzyme then forms an ES compound with the substrate.
3. While coenzyme and substrate are held in proper juxtaposition on the protein surface, the substrate is activated in such a way that it can transfer two hydrogen atoms to the coenzyme, becoming itself an imino acid.
4. The imino acid wanders back into solution, where it reacts spontaneously with water to form the two known end-products, pyruvic acid and ammonia. The removal of the imino acid from the protein surface leaves one active group of the enzyme free to combine with another substrate molecule.
5. Meantime the reduced dinucleotide reacts with oxygen to form hydrogen peroxide, which carries the two hydrogens back into solution and away from the enzyme surface. Removal of the two hydrogens reconstitutes the yellow, oxidized coenzyme, which is thus prepared to accept two more hydrogens from the next molecule of alanine which attaches itself to the enzyme. The cyclical nature of this reaction is well expressed in the following diagram, adopted from Baldwin's *Dynamic Aspects of Biochemistry*.



Each reaction is indicated by a pair of curved arrows which touch each other, the substances written at the blank ends reacting to form the substances at the pointed ends. Clearly the central pair, undergoing a reversible change, can function again and again, reacting first with the alanine and then with oxygen. The alanine and the reduced dinucleotide are in turn hydrogen donors, while the oxidized dinucleotide and then the oxygen act as hydrogen acceptors.

The above outline describes in general terms the course of many different enzymic oxidations. The hydrogen accepted from the substrate by the coenzyme cannot always be passed on directly to molecular oxygen as with this enzyme. Some reduced coenzymes must donate hydrogen to some other type of coenzyme, which will in turn pass it on to oxygen. But the reaction is always of the cyclical type indicated above, involving a specific protein and a coenzyme which can be reversibly reduced and oxidized.

Integrated Enzyme Activity. In order to study the properties of a given enzyme, it has been necessary to separate it not only from the cytoplasmic fabric of the cell itself but from other enzymes. It might have been argued, and indeed it was, that the properties which were found under such unnatural conditions might bear no relation to those exhibited by the catalyst in its normal environment in the cell. Fortunately this has proved not to be true. In very recent years it has become possible to examine cell material without entirely disrupting its organization. The ultracentrifuge has been used to separate into various fractions the tiny particulate elements distributed through the cytoplasm. Some of these are proving to contain whole groups of enzymes so organized that the isolated particles catalyze an elaborate sequence of chemical reactions in which the end product of one catalysis becomes the substrate of the next. This integrated activity consists, not of new and strange reactions, but of reactions already associated with known cell catalysts. That this orderly sequence of events follows a pattern previously deduced from work with isolated enzymes is one of the triumphs of modern biochemistry.

Suggestions for Further Reading

GENERAL

The *Annual Review of Biochemistry* carries each year several articles on various types of enzymes and gives very full references to current periodicals.

BALDWIN, E., *Dynamic Aspects of Biochemistry*, 2nd ed., Macmillan, New York, 1952.

This book is largely a discussion of enzymes and their properties, and is a mine of specific information.

GREEN, D. E., *Mechanisms of Biological Oxidations*, Cambridge University, Cambridge, 1941.

HALDANE, J. B. S., *Enzymes*, Longmans, Green, New York, 1930.

This is a good general discussion of enzyme properties as they were known in 1930.

NORTHROP, J. H., KUNITZ, M., and HERRIOTT, R. M., *Crystalline Enzymes*, Columbia University, New York, 1946.

In this small book the authors have assembled the results of their own investigations with proteolytic enzymes.

SUMNER, J. B., and MYRBÄCK, K. (eds.), *The Enzymes*, Academic, New York, 1950.

The four volumes of this monumental work contain detailed reports on many different enzyme systems, each written by a specialist in the field.

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IMPORTANCE OF SPECIAL GROUPS IN ENZYME PROTEINS

Sulfhydryl Group

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Tyrosine

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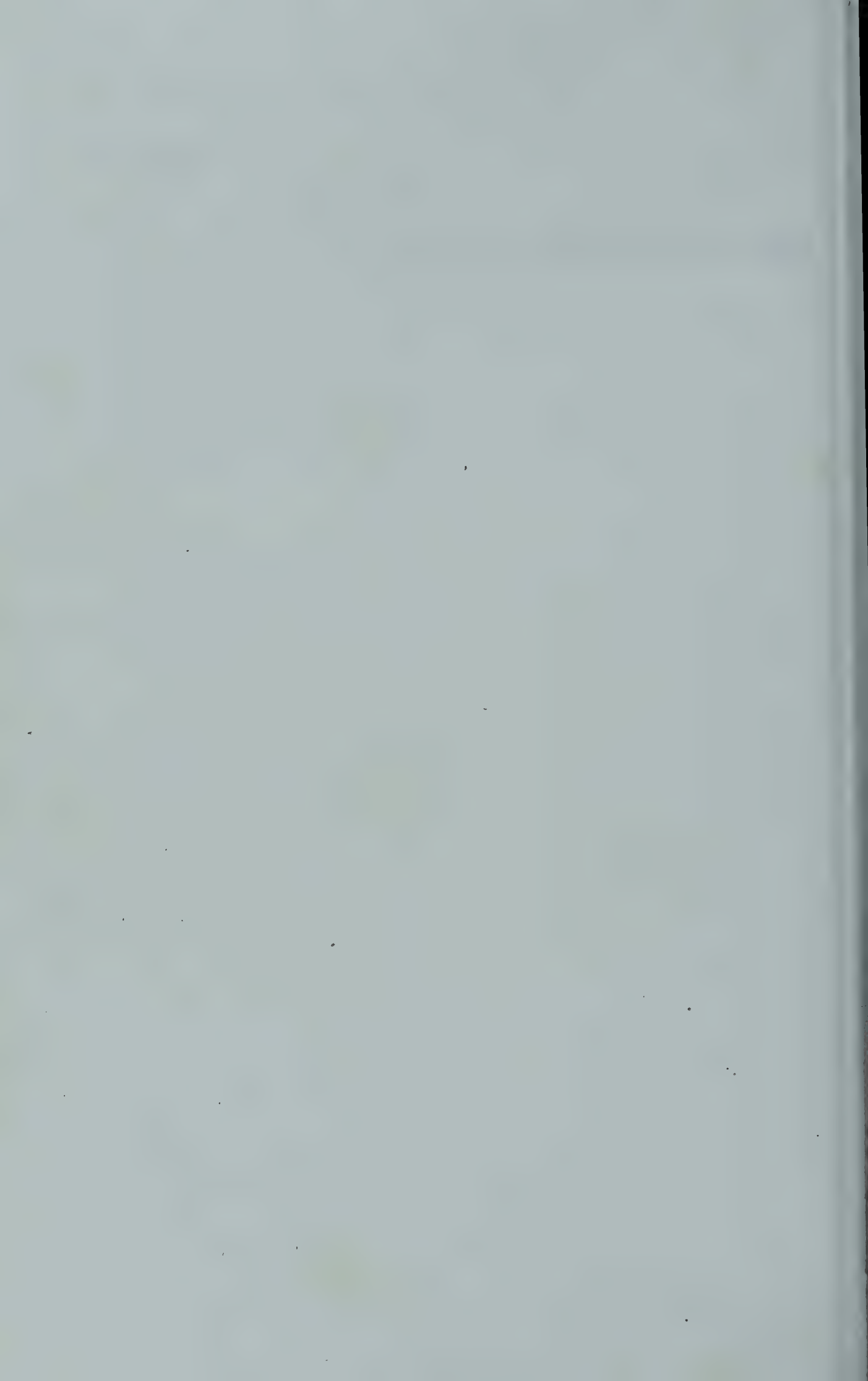
Study Questions

1. What was Liebig's idea of the nature of enzyme action? Compare this with the modern theory. What was Pasteur's theory?
2. What did Buchner contribute to our understanding of the nature of enzymes?
3. What is the "turnover number" of an enzyme?
4. Indicate three different procedures which have been used to render an enzyme available to extracting solvents.
5. What are the various types of specificity exhibited by enzymes? Give samples of each.

6. How is the rate of an enzyme action influenced by changes in (a) temperature, (b) enzyme concentration, (c) substrate concentration?
7. What is a pro-enzyme? Name one and tell how it is activated.
8. What inorganic ions are frequently essential to an enzyme action? What function has been ascribed to them?
9. What is the special characteristic of the oxidizing enzymes?
10. What evidence is there that D-amino acid oxidase acts by reduction of a flavin compound?
11. What is the exact function of an oxidizing coenzyme? of the enzyme protein?
12. How are enzyme inhibitors used in tracing the course of an enzymic reaction? Name three inhibitors. Which is a specific inhibitor of respiration?
13. What evidence was there for the formation of an enzyme-substrate compound before it was possible to identify such complexes spectroscopically?
14. What is the "Michaelis constant"?

Part III

Intermediary Metabolism



The Methods Used in the Study of Intermediary Metabolism

In the study of the intermediate processes of metabolism we have to deal, not with complex substances which elude ordinary chemical methods, but with simple substances undergoing comprehensible reactions.

F. G. HOPKINS: *The Dynamic Side of Biochemistry* (1913)

In the cells of living organisms the foodstuffs undergo profound chemical changes. They may be degraded to carbon dioxide and water and other excretory compounds or the products of their partial breakdown may be used synthetically. All of these changes taken together constitute the *intermediary metabolism* of the cell. Since the transformations follow each other in quick succession, few of the intermediates ever appear in the normal organism in such concentrations as to be measurable. This has made it necessary to approach the problem in all sorts of indirect ways, and for this purpose many different methods have been evolved through the years. Before going on to consider the results of experiments on intermediary metabolism it will be helpful to review briefly some of the more important of these methods, and to learn their terminology.

Experiments with Whole Organisms

The earliest studies of metabolic relationships in animals consisted merely of analyses of food, excreta, and blood. A short time after ingestion of a meal high in starch the concentration of glucose in the blood was found to rise far above its fasting level, indicating preabsorptive hydrolysis of the polysaccharide. Urine collected during the twenty-four hours following a meal of liver, which is rich in nucleoproteins, proved to contain a higher concentration of uric acid than usual. This pointed to the limited oxidation of the purine bases to the closely related uric acid (see p. 194), rather than to their complete oxidation to such small molecules as carbon dioxide and urea. Such experiments as these, carried out with normal organisms, normally nourished, give at best only the beginning and the end of the metabolic story. To elucidate the intervening chemical steps it was necessary to devise other methods.

EXPERIMENTS WITH ABNORMAL ORGANISMS

Probably no single abnormality has contributed more to our knowledge of the intermediary metabolism of animals than has the disease known as diabetes. It usually arises in human patients from the inability of certain cells in the pancreas to secrete insulin. Lack of insulin makes it impossible for the diabetic to metabolize glucose and as a result any glucose which is ingested or which is formed in the body from other molecules, is promptly excreted in the urine. It is possible to induce an experimental diabetes in animals by removal of the pancreas or by injection of alloxan or of phlorizin. When such an animal is given a diet consisting only of protein, it continues to excrete large quantities of glucose in the urine, indicating that the body is transforming protein into carbohydrate. Similar experiments have been carried out with individual pure amino acids replacing protein in the diet of the diabetic animal. They have shown that while certain of the amino acids can be transformed into glucose, others apparently are not so transformed, since their ingestion does not give rise to excretion of glucose in the diabetic.

Other organs than the pancreas may be removed, with more or less profound effects on the animal. Various of the glands, such as the thyroid, the pituitary, or the adrenals have been removed in attempts to determine the parts played in metabolism by their respective secretions. Hepatectomy, or removal of the liver, though a difficult and drastic operation, has been accomplished in such a way that the animals have survived for a few days. Under such circumstances, substances which are normally transformed in the liver cannot be dealt with and either pile up in the blood or appear as abnormal constituents of the urine. This is true, for example, of ammonia which arises from metabolism of the amino acids. Its concentration in blood and urine is normally very low, but following hepatectomy both blood and urine contain increasing amounts of ammonia. Other substances normally formed in the liver cannot be synthesized and the urine of the hepatectomized animal therefore lacks such normal constituents. Urea, for example, is normally synthesized in the liver from the ammonia referred to above, but an animal which lacks its liver soon ceases entirely to excrete urea.

USE OF ABNORMAL DIETS AND CULTURE SOLUTIONS

Another method of attacking the metabolic problem is to make use of normal organisms but to furnish them a diet which is abnormal in one way or another. It may be unduly enriched in some test substance, as when fats are fed exclusively or in great excess, to determine how the body deals with them. Or the diet may include some unnatural substance closely related to a natural one. One of the most fruitful experiments

of this kind was performed early in the century by Knoop.¹ He fed fatty acids in which a phenyl group had been introduced at the end of the carbon chain, as in γ -phenylbutyric acid, $\text{C}_6\text{H}_5\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—COOH}$. Such a substance might be expected to react in the body more or less as do other fatty acids, but since cells do not oxidize a benzene ring it would yield an aromatic and therefore recognizable excretory product. After feeding a whole series of such substituted acids, Knoop recovered from the urine a corresponding series of detoxication products clearly related to the original acids. These results will be considered in detail in the chapter on lipid metabolism.

Experiments with Individual Organs

Because the intact animal or plant is so complex a mechanism with so many interacting nervous and chemical controls, it has been fruitful to study the metabolism of isolated organs and tissues. One method of doing this with animal tissue is known as *perfusion* of the organ. This consists in furnishing an organ with an experimental, independent circulation by pumping some suitable fluid repeatedly through the blood vessels. This may be done with a minimum of injury if the organ is left in place in the anesthetized animal, but is also done after complete removal of the organ from the body. An isolated frog heart has been kept beating for as long as thirty-three days when perfused with a fluid containing only glucose, oxygen, and inorganic salts. To such a solution various substances under investigation may be added. After a time, analysis of a small sample of the perfusion fluid will tell whether or not the organ is using the added substance and may show the presence of some new constituent derived from the added one. Thus, for example, it is clear that an isolated heart makes use of glucose because this substance slowly disappears from the perfusion fluid. When a liver is perfused with a solution containing pyruvic acid, alanine appears as the pyruvic acid disappears, indicating a transformation of the keto acid to the corresponding amino acid. Similarly it has been shown that a perfused kidney forms ammonia from amino acids.

The fluid used for perfusion may be blood from a similar animal to which an anticoagulant has been added, or it may be one or another of the physiological saline solutions. A solution of the chlorides of sodium, potassium, and calcium is known as Ringer's ² solution in honor of the man who first discovered that perfusion with this simple solution keeps an isolated heart beating. Various adaptations of his original formula have

¹ Dr. Franz Knoop (1875–1946) published his early papers from the University of Freiburg, but after 1928 he was Professor of Physiological Chemistry and Director of the Institute of Physiological Chemistry at the University of Tübingen.

² Sydney Ringer (1835–1910) was an English physician with an active practice who yet managed to carry out a good deal of physiological research. His medical teaching was done at University College, London.

been made to render the fluids suitable for use with organs from animals of different species. They have found a wide use not only in perfusion experiments, but in those with tissue slices which will be considered presently.

One animal tissue which lends itself particularly well to experimentation outside the body is striated muscle. A leg muscle, for example, suspended in a damp atmosphere can be stimulated electrically to contract until it is fatigued. It gains the energy for contraction by metabolizing its store of glycogen, and much of our present understanding of carbohydrate metabolism is based upon the fundamental studies made by Fletcher and Hopkins using isolated frog muscles.

In plant experiments isolated leaves are often used as experimental material, as in those studies which proved that, although nicotine may make up as much as 8 per cent of the dry weight of the tobacco leaf, it is synthesized exclusively in the roots. When leaves which were accumulating nicotine were detached from the plant the accumulation ceased abruptly, but began again when the leaves were cultured in sand and allowed to form roots. Similar experiments with excised tobacco roots have indicated some of the intermediates in biosynthesis of nicotine by furnishing in a nutrient solution some supposed precursor and determining whether or not it leads to nicotine synthesis.

Tissue Slice Experiments

The great disadvantages of the perfusion method are that it requires the use of fairly large animals, and that a single organ can be used for only a comparatively short time, and so for a limited number of observations. However, since about 1925, the development of manometric micro methods has made it possible to carry out with either plant or animal tissues experiments of similar import using only a few milligrams of material. These methods were first extensively used and adapted by Warburg³ in Berlin, and by Dixon⁴ at Cambridge.

If a slice of tissue is cut not more than 0.3 mm. in thickness, the cells inside the outermost layers are undamaged and constitute the greater part of the slice. Such a thickness permits ready diffusion inward of oxygen and foodstuffs if the tissue is immersed in a fluid containing these substances. Under these conditions the undamaged cells of a tissue slice will carry on their normal metabolic processes for several hours, respiring, and

³ Otto Warburg (1883–) is one of the outstanding biochemists of the present day. As Director of the Kaiser Wilhelm Institute for Cellular Physiology in Berlin he has taken a leading part in the development of many phases of biochemistry, including biological oxidation, photosynthesis, and tumor metabolism.

⁴ Malcolm Dixon (1899–) is Reader in Enzyme Chemistry at Cambridge University. He is particularly known for his applications of physical chemistry to the study of enzymes and his small book on *Manometric Methods* is a classic in its field.

evolving carbon dioxide. Liver slices, for example, will transform added ammonia into urea; kidney slices will form ammonia from amino acids.

As with perfusion experiments, the fluid used to bathe the slices must contain a carefully balanced concentration of inorganic ions. In particular it is essential that the osmotic pressure as well as the ratio of calcium, magnesium, sodium, and potassium correspond to that in the blood of the animal whose tissues are used. Sometimes the solution is buffered with bicarbonate, sometimes with phosphate, sometimes with other buffers. In Table 8-I are given the ionic compositions of several common saline solu-

TABLE 8-I. COMPOSITION OF VARIOUS PHYSIOLOGICAL SALINE SOLUTIONS

Constituents	Normal Mammalian Serum (mg. % ^a)	Phosphate Ringer (mg. %)	Bicarbonate Ringer (mg. %)	Krebs- Henseleit Solution (mg. %)
Na ⁺	320	314	355	327
K ⁺	22	10.3	10.5	23
Ca ⁺⁺	10	7.5	8.8	10
Mg ⁺⁺	2.5	—	—	2.9
Cl ⁻	370	485	466	454
PO ₄ ⁼	10	—	—	11
SO ₄ ⁼	11	—	—	11.4
HCO ₃ ⁻	152	—	152	152
CO ₂	2.5 vol. % ^b	—	2.5 vol. %	2.5 vol. %
Glucose	ca. 100	—	200	200

^a Mg. % means the number of mg. of the constituent per 100 ml. of solution.
^b Vol. % means ml. of gas at STP dissolved per 100 ml. of solution.

tions. As the table shows, the salt solution of Krebs ⁵ and Henseleit corresponds closely with mammalian serum, and is consequently an excellent medium for metabolism of mammalian tissues. The directions for making up the various nutrient solutions usually give the volumes of "isotonic" salt solutions which are to be mixed to give the desired ionic concentrations. The word isotonic in this context means isotonic with mammalian serum. An isotonic solution of sodium chloride is therefore often referred to as *physiological saline*. For sodium chloride, potassium chloride, potassium-dihydrogen phosphate, magnesium sulfate, and sodium bicarbonate, 0.154M solutions are isotonic; for calcium chloride the proper concentration is 0.11M.

The tissue slice technique was first used to study respiration and gas exchange, and for this purpose special manometric apparatus was developed. One of the most widely used of these is the Warburg apparatus which can be used to follow the rate of any reaction which either uses or evolves a gas. Figure 8.1 shows the Warburg constant temperature bath

⁵ See page 351.

with the manometers in place for an experiment. Figure 8.2 shows two views, taken at right angles to each other, of a single manometer with its

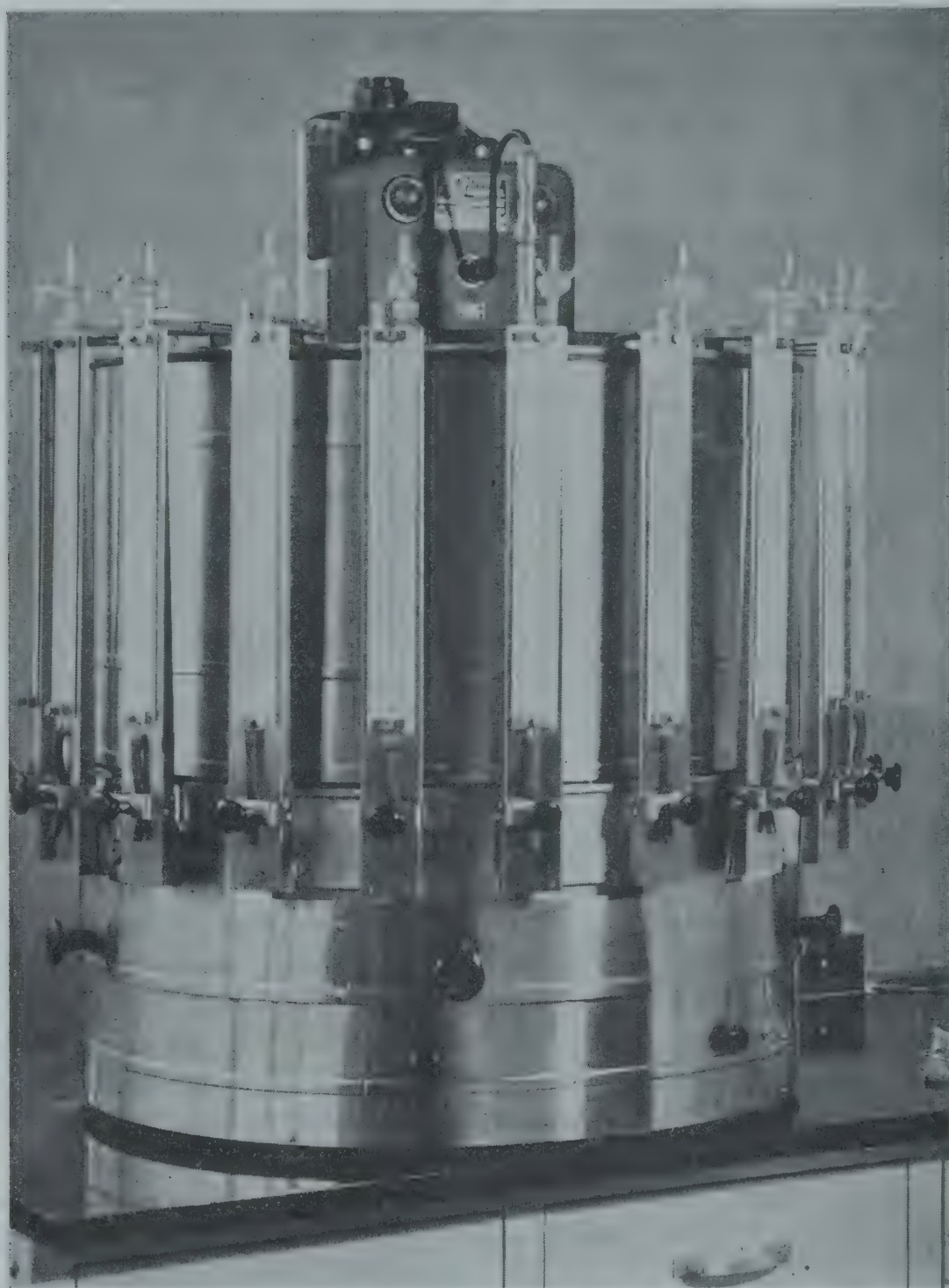


Figure 8.1. The Warburg bath with the manometers in place. The reaction vessels containing the tissue or enzyme are suspended inside the constant temperature bath, and changes in pressure which result from the activity of the enzyme or enzymes are read on the manometers. The Precision Warburg Manometrician is made by the Precision Scientific Company, Chicago. (Photo. Courtesy of Precision Scientific Company.)

attached reaction vessel. These are so designed that the experimental vessel can be lowered into the bath, while the manometer, on which changes in gas pressure are to be read, is held parallel to the outside of the bath.

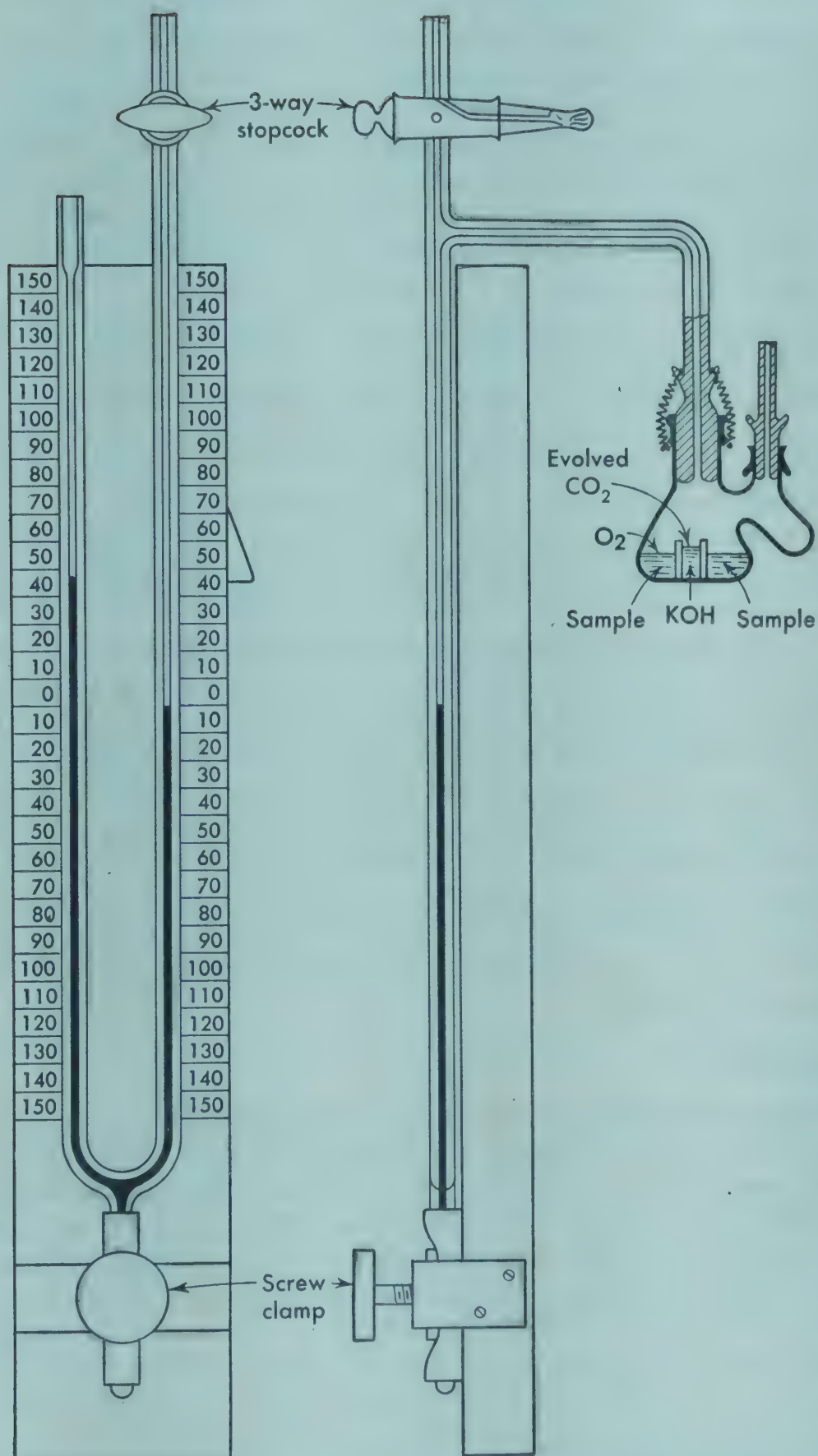


Figure 8.2. Two views of the Warburg manometer and reaction vessel.

A manometer with its attached vessel forms a closed system when the stopcocks are suitably adjusted, the only gas available to the tissue being that enclosed in the vessel and in the top of the manometer down to the liquid level. Each unit is mounted on a movable rack by means of which they can all be gently and continuously shaken to facilitate diffusion in and out of the tissue. The manometer is filled, not with mercury, but with a salt solution known as Brodie's solution. This is of such a density that one atmosphere pressure corresponds to a column 10,000 mm. high. This makes it possible to measure accurately the small pressure changes involved in the metabolism of 100 mg. or less of tissue.

The tissue slice method may of course be used either with or without the Warburg manometric apparatus. Slices may be shaken in suitably buffered saline solutions, to which various possible metabolites have been added, and after a certain length of time the fluid may be examined for derivatives of the added substance. Slices of bird liver, for example, have been shown to synthesize uric acid from added ammonia. At the same time, of course, the living liver cells were carrying on other metabolic activities, using up oxygen to oxidize glucose provided in the nutrient medium, and giving off to the medium carbon dioxide and water. If then such an experiment is carried out in the manometric apparatus the oxygen uptake of the tissues may be measured at the same time if the central cup in the reaction vessel contains strong potassium hydroxide to absorb any carbon dioxide which is evolved. In this case the progressive loss of oxygen, as it is used by the tissue, is reflected in a gradually decreasing pressure inside the vessel. Some tissues and microorganisms are able to exist without oxygen, and their anaerobic metabolism may be followed by replacing the oxygen in the reaction vessel with nitrogen or some other inert gas. Specific examples of various uses of this technique will be referred to from time to time as the results become pertinent in the chapters which follow.

TERMINOLOGY

To express the results of experiments with tissue slices a special terminology has developed. Suppose, for example, the slices of kidney tissue in a single vessel had, in the course of three hours, used up 250 cu. mm. or microliters ($\mu\text{l.}$) of oxygen, corrected to standard temperature and pressure. At the end of the experiment the tissue would be washed and carefully dried to constant weight. The volume of oxygen used, divided by three times the dry weight of tissue in milligrams, would give the volume of oxygen consumed per mg. of dry tissue per hour. If this volume proved to be 23 $\mu\text{l.}$, the result would be expressed: $Q_{O_2} = -23$, the minus sign indicating that the oxygen was used up. In Table 8-II are given figures for the oxygen consumption of a number of representative tissues as measured by the Warburg technique.

As indicated above, experiments are sometimes conducted in an atmosphere of inert gas and in that case the nature of the gas is indicated by a superscript. For example, many tissues form lactic acid from glucose or glycogen if no oxygen is available to them. If this lactic acid forms in a solution buffered with bicarbonate, carbon dioxide will be set free, equivalent in amount to the lactic acid formed. The direct result of such an experiment might be expressed: $Q_{\text{CO}_2}^{\text{N}_2} = +19.1$. This means that, per milligram of dry tissue, per hour, 19.1 cu. mm. of carbon dioxide was set free in an experiment conducted in an atmosphere of nitrogen. The second column of Table 8-II gives representative figures for the carbon dioxide

TABLE 8-II. AEROBIC AND ANAEROBIC METABOLISM OF RAT TISSUES ^a

Tissue	Q _{O₂}	Q _{CO₂} ^{N₂}
Liver	-11.6	+3.3
Kidney	-21	+3.2
Brain (grey matter)	-10.7	+19.1
Retina	-30.7	+88

^a Figures from O. Warburg, *Tumor Metabolism*, translated by F. Dickens, Constable, London, 1930.

evolved by a number of different tissues under these conditions. These figures can be simply transformed into milligrams of lactic acid as follows:

1 mole of carbon dioxide is freed by each mole of lactic acid

22.4 L. of CO₂ at STP corresponds to 90 g. lactic acid

22.4 ml. of CO₂ at STP corresponds to 90 mg. lactic acid

$$\frac{90}{22.4} = 4 \text{ mg. lactic acid corresponds to 1 ml. CO}_2$$

1 μl. of CO₂ is equivalent to 0.004 mg. lactic acid

19.1 × 0.004 = 0.0764 mg. lactic acid formed per hour, per mg. dry weight of tissue in the example given above.

This is often expressed $Q_L^{\text{N}_2} = +0.08$, or $Q_M^{\text{N}_2} = +0.08$, the M standing for *Milchsäure*, the German word for lactic acid.

It should be noted that in all these experiments in which gases are involved, the quantity actually measured is the change in gas *pressure*. This is transformed arithmetically into gas volume by means of a “constant” for each vessel. The derivation of this constant is given in laboratory manuals.

Cell-Free Enzyme Systems

Even a single thin slice of tissue contains a complex system of enzymes whose activities interlock. It is sometimes possible to analyze such a

system by separating it from the cells. With some tissues only part of a group of enzymes is extracted when the cell is chopped or ground with physiological saline or buffer. In others, a tissue so finely ground that cell membranes are ruptured yields a cell-free *brei* or mash which yet contains many enzymes whose activities throw light on the metabolic functions of the tissue itself. Simple extraction of ground muscle with physiological saline dissolves the enzymes responsible for anaerobic formation of lactic acid, leaving behind in the tissue residue those others which bring about oxidation of carbohydrate. Discovery of this simple separation made possible a detailed study of the anaerobic function, uncomplicated by oxidation. Using extracts or breis, enzyme inhibitors may be used to inactivate part of the system, and thus allow some intermediates to accumulate. Or intermediate products may be isolated if the enzyme action takes place in the presence of a reagent which will "trap" them, and so prevent their being destroyed by further metabolic changes. The presence of acetaldehyde as an intermediate in alcoholic fermentation was proved by Neuberg when he added bisulfite to the fermenting press-juice from yeast. As fast as aldehyde formed, it was caught and held as its bisulfite addition product, which was finally present in sufficient quantity to be isolated.

Finally, from one point of view the most satisfactory metabolic experiment is the one which makes use of a single pure enzyme. Here the product of a single reaction accumulates because any enzymes involved in its further metabolism are absent. In such a system single pure substrates may be tested, one by one, and the specific requirements of the enzyme outlined. In some cases it is discovered that an enzyme cannot perform its function except in conjunction with another enzyme action. This happens when an enzyme catalyzes a reaction which uses up energy. Such an endergonic reaction can only proceed if another one, yielding energy, is going on in the immediate vicinity. Such actions are said to be *coupled* and they are most easily discovered in work with pure enzymes. In other words, having carefully analyzed the activities of a tissue into its constituent enzyme actions, we must often perforce put the parts together again to see how they interlock in the cell!

Use of Isotopes

The experiments outlined in the preceding paragraphs have all suffered from one disadvantage or another. If normal organisms and normal nutrients are used, it is impossible to identify the intermediate products in the smooth sequence of reactions. If either the organism or its nutrient is abnormal it is hard to reason from the results obtained to the events to be expected under normal conditions. These disadvantages are of course magnified if an organ is entirely removed from its normal situation or if the whole cellular structure is disrupted as in experiments with

extracts, *breis*, or purified enzymes. In spite of which, many experiments of fundamental importance have been carried out by these very methods, and many just conclusions have been reached.

A new era in metabolic experimentation was inaugurated in 1932 when Urey ⁶ and his colleagues first concentrated deuterium, or heavy hydrogen. Ordinary hydrogen as it occurs in nature consists of a mixture of three isotopes, each having a single charge on the nucleus, but having atomic weights of 1, 2, or 3. These are distinguished as ${}_1\text{H}^1$, ${}_1\text{H}^2$, ${}_1\text{H}^3$. The ratio in which they are found is constant, ${}_1\text{H}^1$ making up 99.99 per cent of the total number of atoms; ${}_1\text{H}^2$, or deuterium, 0.01 per cent; while ${}_1\text{H}^3$, or tritium, occurs naturally only to the extent of about 7 parts in 10^{10} . Whether a sample of hydrogen is free, or is combined in an inorganic or an organic compound, its deuterium content is always found to be 0.01 atom per cent. This means that 1 of every 10,000 molecules of hydrogen chloride is really deuterium chloride; 1 of every 10,000 hydrogen atoms in a fat is heavy hydrogen. Similarly, ordinary oxygen wherever found consists of 99.76 atom per cent of O^{16} , 0.04 atom per cent of O^{17} , and 0.20 atom per cent of O^{18} . Nitrogen occurs, free or in compounds, as a mixture containing besides N^{14} , 0.37 atom per cent of the heavier isotope, N^{15} . The only common elements which do not occur as isotopic mixtures are phosphorus, iodine, and sodium.

In various ways it has been possible to increase greatly the concentration of the less plentiful isotopes, either in a sample of the element or of some compound. For example, when water is electrolyzed, the lighter isotope of hydrogen is freed preferentially, and the remaining water becomes progressively richer in water containing deuterium (D_2O , or heavy water). In one experiment, 20 liters of water from electrolytic cells was further electrolyzed until only 0.5 ml. remained. This water proved to have a deuterium content of 66 atom per cent in contrast with 0.01 atom per cent in ordinary water. Such a sample of heavy water was one of the earliest isotopic compounds used in a biochemical experiment. Its hydrogen is clearly labeled and can be identified wherever it may be found by its abnormal density. The very fact that hydrogen obtained from any part of an animal body contains the normal ratio of isotopes, proves that living cells do not distinguish between them, neither rejecting nor preferring any one. Indeed living organisms can use heavy water in reasonable amounts (up to 25 per cent of the total water intake) as they would use ordinary water. When an animal in whose diet heavy water has been included proves to have an abnormal concentration of deuterium in its

⁶ Harold C. Urey (1893–) was Professor of Chemistry at Columbia University when he made his famous separation of deuterium from ordinary hydrogen. For this work he was awarded the Nobel Prize in Chemistry in 1934. Since 1942 he has been at the Institute for Nuclear Studies at the University of Chicago, except for the war years when he was Director of War Research. His chief interests have been in atomic structure and separation of isotopes.

TABLE 8-III. NATURAL AND RADIOACTIVE ISOTOPES OF BIOCHEMICAL INTEREST

Element	Mass	Abundance (atom %)	Half Life
Hydrogen	1	99.99	
	2	0.01	
	3		12.5 yrs.
Carbon	11		20 min.
	12	98.9	
	13	1.1	
	14		5740 yrs.
Nitrogen	14	99.63	
	15	0.37	
	16		8 sec.
Oxygen	15		126 sec.
	16	99.76	
	17	0.04	
	18	0.20	
Sodium	22		2.6 yrs.
	23	100	
	24		14.9 hrs.
Phosphorus	31	100	
	32		14.3 days
Sulfur	32	95.1	
	33	0.7	
	34	4.2	
	35		87 days
Potassium	39	93.3	
	40	0.01	
	41	6.7	
	42		12.4 hrs.
Calcium	40	96.96	
	42	0.64	
	43	0.15	
	44	2.06	
	45		180 days
	46	0.003	
	48	0.19	
Cobalt	56		72 days
	58		72 days
	59	100	
	60		5.3 yrs.
Iron	54	5.81	
	55		4 yrs.
	56	91.64	
	57	2.21	
	58	0.34	
	59		46 days
Iodine	126		13 days
	127	100	
	130		12.6 hrs.
	131		8 days

body fats, the use of body water in fat synthesis is clearly indicated. Heretofore no such relationship could have been proved, because dietary water as soon as it had been absorbed was inextricably mixed with body fluids, and its hydrogens thus rendered indistinguishable from any others.

Since 1932 natural stable isotopes of many elements have been concentrated and obtained in more or less pure form. During the same years the physicists have prepared artificially radioactive isotopes of nearly every element. This has made it possible to synthesize many different compounds of biochemical importance, each labeled with an abnormal concentration of one isotope or another. Such compounds may be fed or injected into animals, or added to nutrient media of plants or bacteria and their metabolic processes followed by suitable isotope analysis. Table 8-III lists a few isotopes which have been used in biochemical experiments, with the natural abundance of the stable ones, and the half-life time of the radioactive ones.

CHOICE OF ISOTOPES

Various factors will determine whether an experiment can best be carried out with a stable or with a radioactive isotope. The radioactive ones have some special advantages, and some disadvantages for biological work. One great advantage is the simplicity with which their presence may be detected, either photographically, or by means of a Geiger counter. Figure 8.3 shows how readily the presence of a radioactive isotope in tissue may be detected photographically. Radioactive iodine was injected as potassium iodide into a pregnant cow. After twenty-four hours the animal was killed and "radioautographs" were made by placing on an x-ray film slices of the thyroids of mother and fetus. The left hand photographs are ordinary photomicrographs of two thyroid slices, the maternal one in the upper left (A) and that of the fetus below (C). Beside each slice is the picture which resulted when these same slices were allowed to take their own pictures on photographic film. The radiations from the iodine which had largely concentrated in the two thyroid glands caused a blackening of the film, the depth of the darkening being a rough measure of the iodine concentration in different parts of the tissue. Incidentally these pictures illustrate strikingly the extent to which a fetus may drain the maternal iodine stores to build up its own supply of thyroxin.

The Geiger counter is an electronic instrument for detecting directly any ionizing radiation such as is given off by radioactive elements. One form of the apparatus indicates the presence of radiations by flashes of light; another records graphically the amount of radiation received while a third type signals its presence by a clicking sound. An instrument of this latter type can be used with great effect to show how rapidly the food of a nursing mother is transferred to the offspring. A mother mouse is given salt

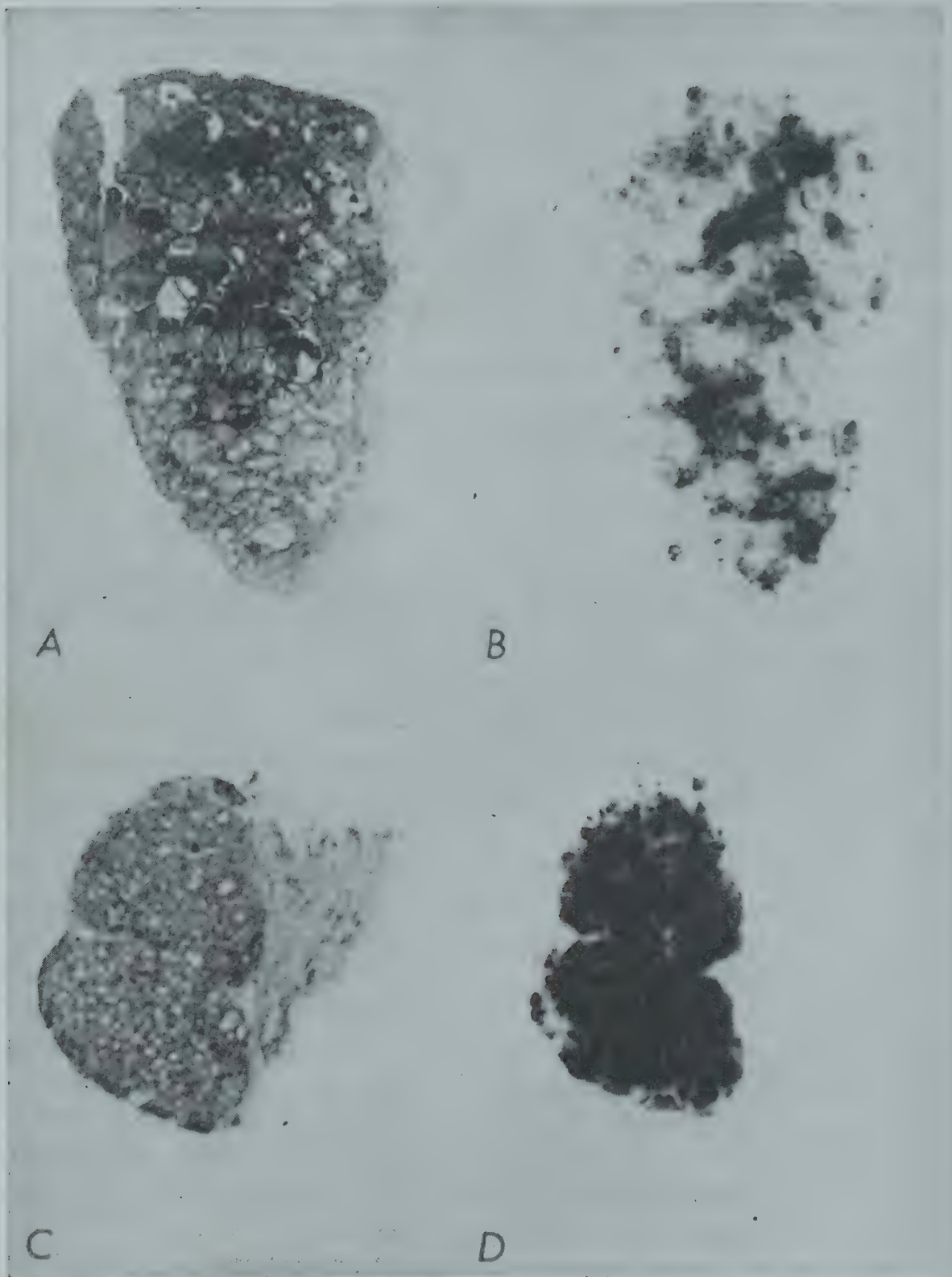


Figure 8.3. Deposition of radioiodine in the thyroids of mother and fetus following injection of radioactive potassium iodide. A. Photomicrograph of the maternal thyroid. B. Radioautograph of the same slice of tissue as is shown in A. C. Photomicrograph of a slice of thyroid from the fetus. D. Radioautograph of the tissue shown in C. (From A. Gorbman et al., *J. Endocrinol.*, 51:546, 1952.)

containing radioactive sodium (Na^{24}). After a few hours if one of the babies is brought near a Geiger counter it sets up a veritable fusillade of clicks, indicating unmistakably the transfer of the radioactive isotope from the mother to the offspring through the milk.

In spite of ease of detection, not all radioactive isotopes are suitable for biochemical investigation. With some elements the half life of the isotope is too short to allow time for synthesis of a compound in the laboratory, or for a normal metabolic transformation in living cells. The usefulness of C^{11} is greatly limited by its having a half life of only 21 minutes, while O^{15} with a half life of 126 seconds is too unstable to be used at all. The character of the radiation emitted may also be a determining factor. Tritium (H^3) is radioactive and has a half life of 12.5 years, but will probably never be widely used because its radiation is so soft that it is extremely difficult to detect. The elements whose radioactive isotopes have so far been most widely used to study the metabolism of organic compounds are radioactive carbon (C^{14}), phosphorus (P^{32}), sulfur (S^{35}), and iodine (I^{126}). These are all relatively simple to prepare, they have convenient half-life times, and they emit radiations which are easy to measure.

Of the elements which make up the major part of the foodstuffs, carbon, hydrogen, nitrogen, and oxygen, it was the stable isotopes which were the first to be used. More recently the available supply of radioactive C^{14} has made it possible to use this long-lived isotope to trace the fate of specific carbon atoms in both plant and animal metabolism. But the radioactive isotopes of nitrogen and oxygen are too short lived to compete with N^{15} and O^{18} and, as already noted, it is easier to determine the concentration of stable deuterium than to detect the soft radiation of tritium.

REPRESENTATIVE ISOTOPE EXPERIMENTS

The usual biochemical isotope technique involves furnishing a plant or animal with a compound having an abnormally high concentration of one or more isotopes, with a view to determining what chemical transformations the compound undergoes in the living cells. It is not normally possible nor desirable to use a compound which contains only the less abundant isotope. Pure heavy water, for example, is toxic to living organisms. Furthermore, it has been shown that at ordinary temperatures pure elementary deuterium reacts much more slowly than hydrogen, and that compounds containing very high percentages of the heavy isotope are oxidized enzymically at about half the ordinary rate. It is therefore customary to use compounds only moderately enriched in the less usual isotope, and actually this is all that is needed. For example, it has been noted that nitrogen normally contains 0.368 atom per cent of N^{15} . The methods for detection of this isotope are so sensitive that if this percentage

is increased to only 0.371 atom per cent, it is possible to detect the difference, even if the amount of nitrogen available for analysis is less than 1 mg.!

Before using a labeled compound to study its fate in metabolism, it is necessary to make sure that the isotope is stably bound. This fortunately is true of nitrogen linked to carbon as in the amino acids, and so N^{15} has been incorporated into many amino acids and its metabolic fate determined. For example, labeled glycine was fed to human beings, and the exact rate at which that particular nitrogen was excreted was followed by estimating the isotopic ratio in the urinary urea. In another experiment, serine ($CH_2OH-CH[NH_2]-COOH$) was labeled with N^{15} and also with C^{13} in the carboxyl group. This was fed to rats, together with some sodium benzoate. It will be recalled that benzoate is detoxicated by conjugation with glycine, and thus gives rise in the urine to benzoyl glycine, or hippuric acid. In the experiment in question the hippuric acid proved to contain both N^{15} and C^{13} in abnormal amounts, thus proving the conversion of serine to glycine in the animal body.

But when the isotope in question is deuterium, its position in a compound must be carefully chosen, for in many positions hydrogen is very unstably held. Most obviously, an acidic hydrogen, ionizing in an aqueous medium, ceases to label the acid from which it came. But other hydrogens are also labile. It has been shown that organic hydrogen bonds may be grouped into three classes. In the first, hydrogen is *labile* and exchanges freely with the aqueous medium. This class includes the hydrogen of carboxyl, hydroxyl, primary amino, or aldehyde groups, as well as hydrogen attached to a carbon which itself holds a carbonyl group. Deuterium in any of these positions would be no label at all. Hydrogen forms a few *semilabile* bonds which exchange only on prolonged boiling in acid. This is true of all the hydrogens of glycine, and of those *ortho* to the hydroxyl group in tyrosine. Finally, hydrogen bound directly to carbon is usually stable. As noted above, this is not true of hydrogen adjacent to a carbonyl group, nor of any hydrogen in glycine. But these exceptions leave a large field for experimentation. Deuterium has been added to double bonds in fat acids, for example, to give saturated compounds which can be used to investigate the fate of such compounds in metabolism. If the deuterium is found later in other acids in the animal's body fats, it is safe to conclude that it has come there as a result of the metabolic transformation of the original fatty acid. In another type of experiment, phenylalanine was labeled by introducing deuterium into the ring. This acid was fed to rats and later it was proved that the tyrosine ($HO-C_6H_4-CH_2-CH[NH_2]-COOH$) in the body proteins of those animals was high in deuterium. This established the metabolic transformation of phenylalanine to tyrosine.

Isotope experiments are also being used in studies of plant metabolism. For example, when green algae metabolize in an atmosphere which contains $C^{14}O_2$ the compounds which they form in the course of photosynthesis are all labeled with the radioactive isotope. These compounds can then be extracted from the plant and separated by paper chromatography, after which a radioautograph of the chromatogram can be made by placing the sheet of paper on a photographic plate. Dark spots on the plate indicate individual compounds which contain the radioactive carbon. The methods which have been developed make it possible to identify intermediate metabolites when they are present in concentrations of less than $10^{-6}M$. in samples weighing only a few milligrams. It is through such experiments as these that the intermediate steps in the photosynthetic process are gradually being elucidated.

The outstanding advantage of the isotope technique is that it can be used in studies of normal organisms, under normal conditions. It has yielded proof of the truth of some old theories and has completely disproved others. It has yielded answers to some problems which could have been solved by no previously known experimental technique. It is at the moment the most promising method of approach to many other problems which still await solution.

Suggestions for Further Reading

MANOMETRIC METHODS

- DIXON, M., *Manometric Methods*, 3rd ed., Cambridge University, Cambridge, 1951.
 KREBS, H. A., "The Use of ' CO_2 -Buffers' in Manometric Measurements of Cell Metabolism," in *Carbon Dioxide Fixation and Photosynthesis*. No. V of the Symposia of the Society for Experimental Biology, Academic, New York, 1951.
 PERKINS, J. J., "Barcroft-Warburg Manometric Apparatus," *Ind. Eng. Chem., Anal. ed.*, 15:61, 1943.
 UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F., *Manometric Techniques*, Burgess, Minneapolis, 1945.

ISOTOPIC TECHNIQUES

- MENDEL, J. L., and VISSER, D. W., "Studies on Nitrate Reduction in Higher Plants," *Arch. Biochem. Biophys.*, 32:159, 1951.
 RITTENBERG, D., and SHEMIN, D., "Isotope Technique in the Study of Intermediary Metabolism," in *Currents in Biochemical Research*, Green, D. E. (ed.), Interscience, New York, 1946.
 SCHOENHEIMER, R., and RITTENBERG, D., "Study of the Intermediary Metabolism of Animals with the Aid of Isotopes," *Physiol. Revs.*, 20:218, 1940.
 The early Schoenheimer papers in the *J.B.C.*, 111 and 127 give a clear discussion of the method, its limitations and techniques.

Study Questions

1. What are the advantages and disadvantages of the perfusion technique for studying intermediary metabolism?
2. Why is it necessary to use for perfusion experiments a solution containing inorganic ions? What are the chief ions included?
3. How have diabetic animals been used to study carbohydrate precursors?
4. Describe briefly the technique known as the "tissue slice" method, telling exactly how you would proceed to find out whether or not a given tissue could oxidize amino acids.
5. What does it mean when it is stated that a certain tissue has a $Q_{O_2} = -18$? that another has a $Q_{CO_2}^{N_2} = 12.5$?
6. What are the advantages of experiments with cell-free enzyme solutions?
7. What is the great advantage of work with molecules labeled with rare isotopes?
8. What are the two types of isotope available for experimental work? Under what conditions is one to be preferred to the other?
9. Name five isotopes which have been especially important in biochemical work, giving their atomic weights, and stating whether or not they are stable.
10. What is a radioautograph? How could one be used to indicate the distribution of radioactive phosphorus in a plant which had grown in a solution containing phosphate salts labeled with the radioactive isotope?

Transportation Systems

With respect then, to the use of respiration, it may be affirmed that an aërial something essential to life, whatever it may be, passes into the mass of the blood.

JOHN MAYOW: *De Respiratione* (1668)

All those plants and animals which are high enough in the evolutionary scale to have special organs dedicated to the performance of specific, limited functions have also, of necessity, some sort of transportation system. This consists of a fluid of greater or less complexity, moving from one part of the organism to another, usually through vessels which carry it to all the tissues. In animals the main circulatory system is a closed one in which the blood moves out from the heart and returns to the heart to be despatched again to the peripheral tissues by way of the lungs. In plants the roughly analogous system is made up of the phloem and the xylem through which the plant saps carry food and other substances from one area of the plant to another.

Blood

The circulating fluid in the higher animals is really a tissue composed of cells floating in a complex solution which is largely colloidal. This moving tissue performs a variety of important functions. It carries food and oxygen to all the cells of the organism and removes the waste products of their metabolism; it transports the hormones from the site of their secretion in the endocrine glands to the tissues which use them; it helps to maintain the water balance by controlling the flow of water into the tissue spaces; it plays a large role in the control of the pH of the body and it regulates body temperature by cooling such tissues as the muscles in which heat is produced and warming the surface areas from which heat is lost. Of the various animal circulatory fluids mammalian blood has been most extensively investigated, hence blood chemistry as presented in the following pages is largely the chemistry of human and closely related bloods. The human circulatory system is represented diagrammatically in Figure 10.4, on page 328.

COMPOSITION OF MAMMALIAN BLOOD

If blood is drawn into a tube or syringe containing an anticoagulant, all the cells or *formed elements* may be spun down in the centrifuge, leaving a clear, yellowish supernatant fluid, the blood *plasma*. In the absence of an anticoagulant drawn blood sets in a clot, from which there is slowly squeezed out a pale yellow liquid called *serum*. Coagulation is a complicated process which results from a series of reactions in which ionic calcium and several of the plasma proteins are involved. The chief result is the transformation of the soluble protein fibrinogen into fibrin which gels to form the actual clot. Thus the main difference between serum and plasma is that the latter contains fibrinogen and the former does not. The common anticoagulants, sodium oxalate and sodium citrate, act by precipitating the calcium ion, thus interrupting the train of events which leads to formation of fibrin.

The cells which comprise the formed elements in mammalian blood are of three kinds, the red blood corpuscles, the white blood cells, and the platelets. The platelets have a special function in helping to initiate blood clotting; the white cells, of which there are several different kinds, serve chiefly as a protection against pathogenic bacteria which they engulf and digest. Since the white cells are nucleated and move about freely, their chemistry is that of any unicellular organism and needs no special comment. Human red cells on the other hand have no nuclei and are essentially little passive containers in which hemoglobin is carried through the circulatory system. The chemical processes which they mediate are unique and will be considered shortly.

The plasma is an extremely complex solution containing proteins, amino acids, carbohydrates, lipids, and salts as well as vitamins, hormones, and enzymes. It is probable that it contains trace amounts of many substances which are still undetected, but the concentration range of most of its constituents is now known. Roughly, about 92 per cent of the plasma is water, nearly 7 per cent is protein, less than 1 per cent is salts, and approximately 0.1 per cent is glucose. Other substances which are present in small amounts include the lipids, which occur in widely varying concentrations, organic acids, and several substances which are grouped together as "non-protein nitrogen compounds." In Table 9-I are listed the most abundant constituents of human plasma or serum. It should be noted that except for the proteins the concentrations are so low that they are reported as milligrams per cent, that is, milligrams of the constituent per hundred milliliters of fluid.

In very recent years Cohn¹ and his associates at Harvard have sepa-

¹ Edwin J. Cohn (1892-1953) was Professor of Physical Chemistry at the Harvard Medical School. His chief interest had been in the physical chemistry of the amino acids and more recently in the elegant methods which he had devised for the isolation of electrophoretically homogeneous proteins from blood serum.

TABLE 9-I. COMPOSITION OF NORMAL HUMAN PLASMA ^{a, b}

	Average or Representative Value (mg./100 ml.)	Range (mg./100 ml.)
Inorganic constituents		
Water	93,600	92,400-94,400
Chloride	365	355-381
Sodium	316	300-330
Bicarbonate (as NaHCO ₃) ^c	226	205-280
Potassium	17.2	12.1-25.4
Phosphate (as P) (inorganic) ^d	3.2	2.6-5.4
Calcium (serum)	10	8.2-11.6
Silica (as SiO ₂) (whole blood)	9.0	
Sulfur, total nonprotein	3.38	2.95-3.75
Magnesium (serum)	2.0	1.7-2.3
Zinc	0.21	0.12-0.48
Copper	0.12	0.086-0.161
Iron	0.105	0.028-0.210
Carbohydrates		
Glucose, fasting capillary whole blood	93	
Pentose, total	2.55	
Polysaccharides (serum) (as hexose)	102	73-101
Nonprotein nitrogen compounds		
Amino acids, total (as N) (ninhydrin method)	4.1	3.4-5.5
Creatine (serum)	1.07	0.76-1.28
Creatinine (serum) (male)		1.05-1.65
Uric acid (serum)	4.0	2.9-6.9
Urea (male)	27.1	
Lipids		
Fatty acids (as stearic acid)		200-450
Fats, neutral		0-150
Cholesterol, free and esterified		150-260
Phospholipids		150-250
Proteins ^e		
	g./100 ml.	
Albumin	4.04	
α ₁ -Globulin	0.31	
α ₂ -Globulin	0.48	
β-Globulin	0.81	
γ-Globulin	0.74	
Fibrinogen	0.34	
TOTAL	6.72	

^a Data in this table are adapted from the full tables given in H. A. Krebs, "Chemical Composition of Blood Plasma and Serum," *Annual Review of Biochemistry*, 19:409, 1950.

^b Note that the figures for silica and glucose are for whole blood. Except where indicated all others are for plasma.

^c All bicarbonate ion present is reported as NaHCO₃.

^d Does not include organic acid-soluble phosphate esters.

^e The six groups listed are the main fractions into which human plasma proteins are separated by electrophoresis. Each group undoubtedly is a mixture.

rated the complex mixture of plasma proteins into twenty-five or more individual fractions. Quantitative data on some of these fractions are assembled in Table 9-II, which shows how various physiologically active substances are distributed and lists some of their presumed functions. Work in this field is only just beginning and anything like a complete

TABLE 9-II. SOME COMPONENTS OF HUMAN PLASMA PROTEINS ^a

Substance	Properties	Assumed Functions	Estimated Conc. (g./100 ml. plasma)	Electrophoretic Fraction
Fibrinogen	Converted to fibrin	Clotting	0.27	
Antihemophilic globulin	Clots hemophilic blood	Clotting	v. little	
Nonclottable protein	Insol. at low temps.		0.01	
Immune γ -Globulin	Antibodies	Immunological	0.74	γ -Globulin
Immune euglobulins	Typhoid agglutinins	Immunological	v. little	β - and γ -Globulins
Enzymes		Metabolic	0.001	α - and β -Globulins
β -Pseudoglobulin	Combines with Fe and Cu	Solubilization and transport of serum components	0.17	α - and β -Globulins
Glycoproteins	Combined with carbohydrates		0.08	α - and β -Globulins
Lipoproteins	Combined with steroids and carotenoids	Solubilization and transport	0.04	α - and β -Globulins
Bilirubin-containing proteins			0.003	α -Globulin
Albumin		Osmotic regulation	3.35	Albumin

^a These data come from a table in H. A. Krebs, "Chemical Composition of Blood Plasma and Serum," *Annual Review of Biochemistry*, 19:409, 1950. The original figures are from papers by Cohn.

quantitative analysis of the plasma proteins is still in the future. These data are included here as an indication of the vast complexity of blood plasma, and particularly of its protein fraction.

Regulation of the water balance of the body is primarily the function of the plasma proteins and especially of the large albumin fraction. Although the greater part of the osmotic pressure of the blood is due to the salts, these substances pass too freely through cell walls to have an appreciable effect on the distribution of water. But as long as the plasma contains its normal complement of the hydrophilic albumin, its small but constant osmotic pressure acts as a check on the capillary pressure which is tending to drive water into the tissue spaces. When for any reason the plasma protein concentration falls below about 3 per cent this control fails and the excessive loss of water into the tissues gives rise to the localized, puffy swelling known as *edema*.

Distribution of Solutes. Certain of the blood constituents are unequally distributed between cells and plasma, as is shown in Table 9-III. Particularly striking is the fact that nearly all the potassium ion is concentrated in the cells while a major portion of the sodium is found in the serum. Why these simple cations do not diffuse freely is not known. The various unequal distributions are undoubtedly due in part to a very complex Donnan effect, but it has not yet been possible to work out the relationships mathematically in terms of a Donnan equation. The one case in which there is known to be a reciprocal relation between two ion con-

TABLE 9-III. DISTRIBUTION OF CERTAIN SOLUTES BETWEEN CELLS AND PLASMA IN NORMAL HUMAN BLOOD ^a

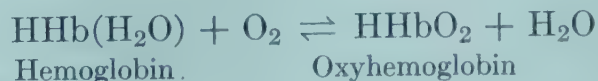
	Moles per 1000 g. H ₂ O	
	Serum	Cells
Protein	0.002	0.007
Urea	0.007	0.007
Glucose	0.004	0.004
Chloride	0.111	0.074
Inorganic phosphate	0.002	0.002
Calcium	0.003	0
Magnesium	0.001	0.003
Potassium	0.004 *	0.135 *
Sodium	0.145 *	0.027 *

^a Data from J. P. Peters, *Body Water*, Thomas, Springfield, Ill., 1935. The starred figures are revised values kindly provided by Dr. Peters.

centrations involves, not a negative and a positive ion as in a simple Donnan equilibrium, but the two negative chloride and bicarbonate ions. Whenever carbon dioxide entering or leaving the blood changes the bicarbonate ratio in cells and plasma, there is an accompanying inverse change in the chloride ratio. This process, known as the chloride shift, takes place twice in each complete circuit of the blood (see p. 312).

HEMOGLOBIN

The ability of the blood to carry large volumes of oxygen depends almost entirely on the presence inside the red blood cell membrane of the pigment hemoglobin. In solution this compound, which contains iron in the ferrous state, readily forms a loose molecular compound with oxygen without changing the valence of the iron. When the oxygen pressure is lowered the complex dissociates rapidly and again sets free its oxygen. It is therefore able to acquire oxygen in the lungs where the oxygen pressure is about 100 mm. of mercury, and to release it in the tissues where the oxygen pressure averages less than 40 mm. Recently it has been shown that this reversible oxygenation takes place only in the presence of water, which is believed to occupy in the reduced molecule the place held by oxygen in oxyhemoglobin. Allowing HHb to stand for hemoglobin to emphasize its acid nature, the two reactions may be formulated:

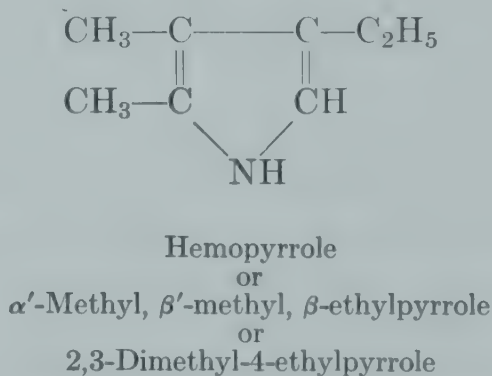
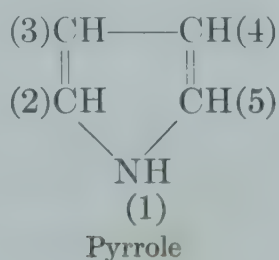


Structure of Heme. Hemoglobin is a chromoprotein which is easily hydrolyzed by dilute acetic acid to set free a colorless protein, globin, from its colored, iron-containing prosthetic group, *heme*. The protein contains a high proportion of histidine, is soluble in water and in dilute acid and

alkali, and belongs to the histone group. The heme is one of a small group of closely related colored compounds which are of unique importance in the living economy. For example, the structure of chlorophyll is extraordinarily like that of heme, while combinations of heme with various proteins give rise to some of the most fundamental and ubiquitous of the oxidizing enzymes as well as to a large group of vertebrate and invertebrate hemoglobins.

It has long been known that when a small quantity of blood is allowed to evaporate in the presence of acetic acid and a trace of sodium chloride, characteristic brown crystalline rods appear. These consist of a chloride of heme to which the name *hemin* is given. It was the exhaustive examination of this compound by many outstanding organic chemists, and especially by Küster,² Willstätter,³ and Hans Fischer⁴ which finally established the structure of heme.

Treatment of hemin with hydriodic acid and acetic acid gives rise as chief products to several differently substituted pyrrole bases. The formulas below, for pyrrole itself and for one of these products of the reductive decomposition of hemin, indicate how the atoms of the pyrrole ring are numbered. In later formulas, in accordance with accepted usage, the carbons and hydrogens of the pyrrole rings are omitted.



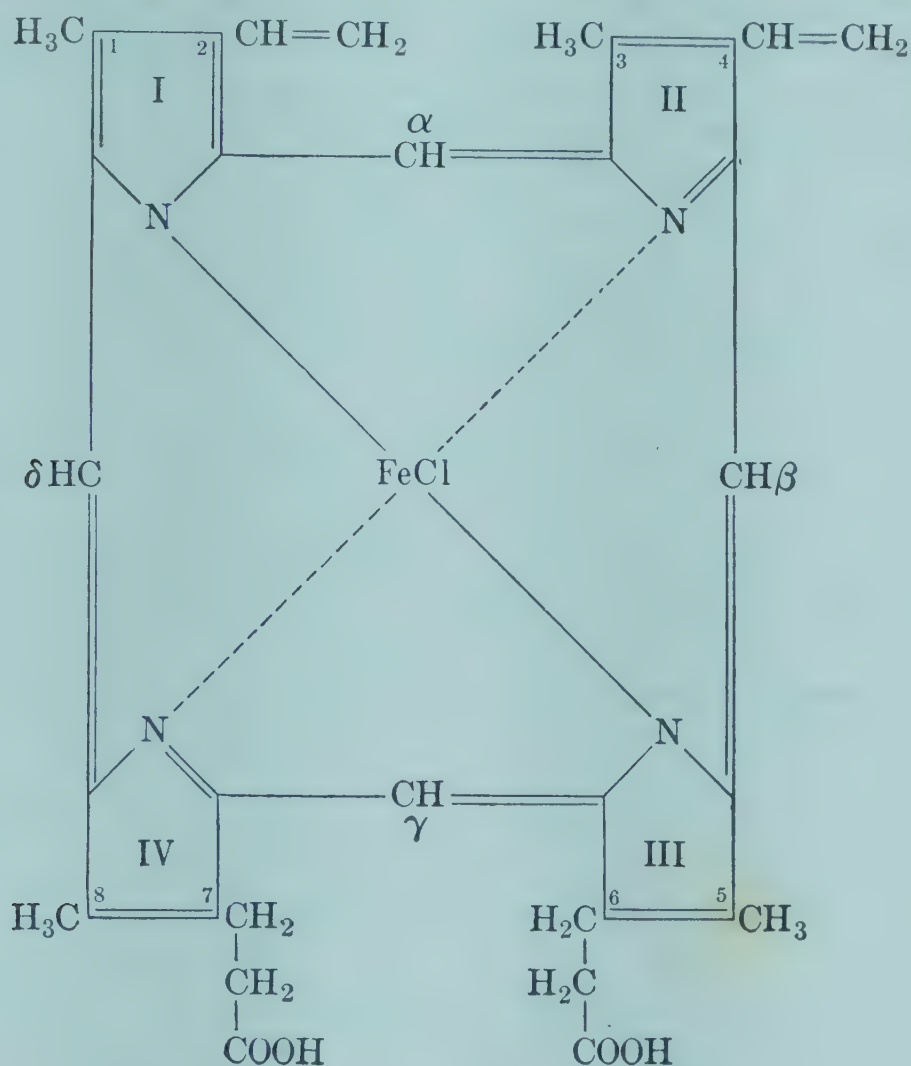
The molecular weight of hemin indicated that four pyrrole rings were probably involved in its structure, which was finally proved by Hans

² William Küster (1863-1929) was Professor of Physiological Chemistry at the Imperial Technical College at Stuttgart. From his laboratory there came a long series of papers dealing with the chemistry of the blood pigments and related compounds. In these he laid the foundations for later work with these complex substances.

³ Richard Willstätter (1872-1942) was Professor of Chemistry at the University of Munich, and although he became Professor Emeritus in 1925 he continued to publish on a wide range of subjects until shortly before his death. His personality and genius attracted graduate students from all over the world, and his school made fundamental contributions to enzyme purification and action and to the chemistry of the plant pigments. He was awarded the Nobel Prize in Chemistry in 1915.

⁴ Hans Fischer (1881-1945) was Professor in the Chemical Institute of the Technical College in Munich. He is best known for his work on the two pigments, heme and chlorophyll, and for his contributions in these fields was awarded the Nobel Prize in 1930. The long strain of the war and the destruction of his Institute led to his tragic death by his own hand.

Fischer's synthesis to be that of an *iron porphyrin*. The large porphyrin ring consists of four pyrrole rings united through methene ($=CH-$) bridges in such a way that eighteen of the inner atoms make up a ring of conjugated double linkages. Obviously the possibilities for resonance are many and the particular distribution of double bonds in the formula



Hemin

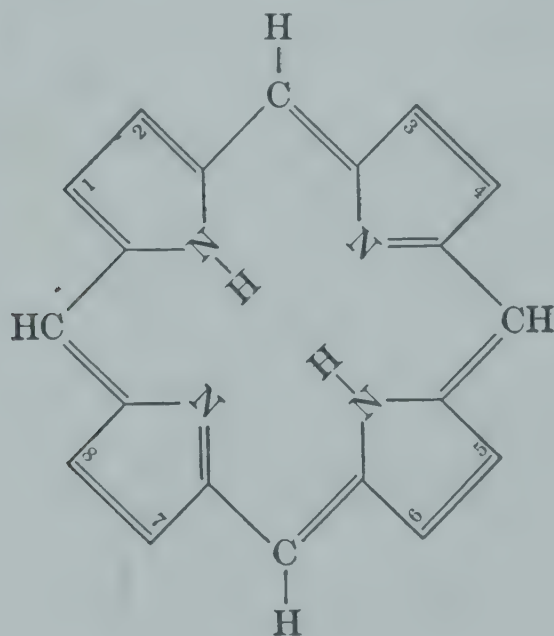
simply serves to give a concrete representation of something for which no single formula can be written. The two types of numbers and the Greek letters are used in differentiating the four rings and the various possible substitution positions.

Plant Hemoglobin. Leguminous plants have an almost unique place in nature in that in the presence of certain soil bacteria they are able to fix atmospheric nitrogen and thus to use it in synthesis. This reaction takes place in nodules formed by bacteria on the roots of the plants. In these nodules hemoglobin has been identified, partly by its absorption spectrum, partly by its reversible reaction with oxygen and partly by the bile pigment-like decomposition products which it forms. Up to the present it has not been possible to prove that it serves a function in symbiotic nitrogen

fixation, but its localization in the nodules certainly suggests that either the plant or the bacteria make some use of its ability to store and to release oxygen.

Nomenclature. The nomenclature of the porphyrins is complicated. In the first place many of the compounds were identified spectroscopically long before their structures were known, and the many very similar names were designed to indicate a complex set of interrelationships. Furthermore there have been recent changes in nomenclature with the result that the same substance appears under more than one name even in current publications. Finally there are still some differences in British and American usage beyond the British use of the diphthong. While the details of nomenclature in this large group of compounds is beyond the scope of such a book as this, it seems wise to give a few guideposts so that anyone dipping into the literature of heme chemistry may not be hopelessly confused at the outset.

A large ring composed of four unsubstituted pyrrole residues united through methene bridges is considered the parent substance of the tetrapyrrole compounds and was named *porphin*. Its various substitution products were then called *porphyrins*. This name is still the one most commonly used, but there is a tendency of late years to drop the middle syllable and to call the derivatives "porphins." In the formula given for the parent compound, porphin, a second common representation of the ring tetrapyrrole compounds is used.



Porphin

Removal of the iron from heme leaves a porphyrin with eight substituents, four methyl groups at positions 1, 3, 5, and 8, two vinyl groups at positions 2 and 4, and propionic acid residues at positions 6 and 7. This compound is known as *protoporphyrin*. If hemin is reduced very gently before removal of the iron only the two vinyl groups react and the product

is therefore identical with protoporphyrin except that the vinyl groups have been replaced by ethyl groups. This compound is known as *mesoporphyrin*. In order to determine the positions occupied by the various substituents in the mesoporphyrin related to heme, Hans Fischer determined to synthesize it. He began by drawing up a table showing that there were fifteen possible arrangements. When later he succeeded in preparing a compound identical with the natural mesoporphyrin it proved to correspond with the ninth one on his list. Hence the mesoporphyrin and the protoporphyrin related to heme are often referred to as mesoporphyrin IX and protoporphyrin IX. The particular variety and arrangement of substituents in protoporphyrin IX seem to be adaptable to many biological purposes, for with only minor variations they are characteristic of all the naturally occurring porphyrins.

Another substance which was important in the elucidation of the structure of heme is *etioporphyrin* which is formed when hemin is vigorously reduced and then subjected to pyrolysis. In these reactions not only are the vinyl groups saturated, yielding ethyl groups, but the propionic acid groups are also transformed into ethyl groups by loss of carbon dioxide. With only two kinds of substituents in the molecule there are but four possible arrangements of the side chains. The one which is present in the etioporphyrin derived from heme proved to correspond with the third one on the original list, hence substances having this arrangement are said to be related to etioporphyrin III.

Complete removal of the unsaturated side chains of hemin can be achieved by bacterial action giving rise to a *deuteroporphyrin*, unsubstituted at positions 2 and 4. From deuteroporphyrin it is possible to prepare another heme derivative, *hematoporphyrin*, with hydroxyethyl groups at positions 2 and 4.

Besides these substances which have been prepared from heme, there are three porphyrins which occur naturally, coproporphyrin I, coproporphyrin III and uroporphyrin I. These substances normally occur in trace amounts only and are believed to be by-products of protoporphyrin synthesis in the body. The compounds with the type I structure are related to etioporphyrin I in which the ethyl and methyl groups alternate around the large ring. The composition of these various porphyrins is given in Table 9-IV.

It was noted above that the iron both in reduced and in oxygenated hemoglobin is in the ferrous condition. But the valence of the iron can be changed if hemoglobin is treated with an oxidizing agent such as potassium ferricyanide. The prosthetic group is then sometimes known as *hematin*, or the difference in the two types of compound may be indicated by the use of the prefixes ferro- and ferri-. Recently it has been suggested that hemoglobin be used for the conjugated protein containing ferrous iron and hemoglobin for the ferric derivative. This latter substance appears in all except the most recent literature as *methemoglobin*, which

TABLE 9-IV. PORPHYRIN COMPOUNDS RELATED TO HEME

	Substituents		
	Positions 1,3,5,8	Positions 2,4	Positions 6,7
Protoporphyrin IX	(—CH ₃) ₄	(—CH=CH ₂) ₂	(—CH ₂ —CH ₂ COOH) ₂
Mesoporphyrin IX	(—CH ₃) ₄	(—C ₂ H ₅) ₂	(—CH ₂ —CH ₂ COOH) ₂
Etioporphyrin III	(—CH ₃) ₄	(—C ₂ H ₅) ₂	(—C ₂ H ₅) ₂
Etioporphyrin I	1,3,5(—CH ₃) ₃ 8—C ₂ H ₅	(—C ₂ H ₅) ₂	6—C ₂ H ₅ 7—CH ₃
Deuteroporphyrin IX	(—CH ₃) ₄	(—H) ₂	(—CH ₂ —CH ₂ COOH) ₂
Hematoporphyrin	(—CH ₃) ₄	$\left(\begin{array}{c} \text{OH} \\ \diagup \\ -\text{CH} \\ \diagdown \\ \text{CH}_3 \end{array} \right)_2$	(—CH ₂ —CH ₂ COOH) ₂
Coproporphyrin III	(—CH ₃) ₄	(—CH ₂ —CH ₂ COOH) ₂	(—CH ₂ —CH ₂ COOH) ₂
Coproporphyrin I	1,3,5(—CH ₃) ₃ 8—CH ₂ —CH ₂ COOH	(—CH ₂ —CH ₂ COOH) ₂	6—CH ₂ —CH ₂ COOH 7—CH ₃
Uroporphyrin I	1,3,5(—CH ₂ COOH) ₃ 8—CH ₂ —CH ₂ COOH	(—CH ₂ —CH ₂ COOH) ₂	6—CH ₂ —CH ₂ COOH 7—CH ₂ COOH

is formed in the body as a result of poisoning by nitrobenzene and other toxic agents. There is some evidence that a very small amount of methemoglobin is normally present in the red cells, but when there is any appreciable amount of this substance the condition is pathological and is referred to as *methemoglobinemia*.

Another hemoglobin derivative which is formed in cases of carbon monoxide poisoning is *carboxyhemoglobin* (HHbCO). In this compound carbon monoxide holds the position normally occupied by oxygen, and as the carbon monoxide is very tenaciously held the hemoglobin ceases to function in oxygen transport.

Heme unites not only with globin but with a large number of other proteins and even with such simple basic compounds as pyridine and am-

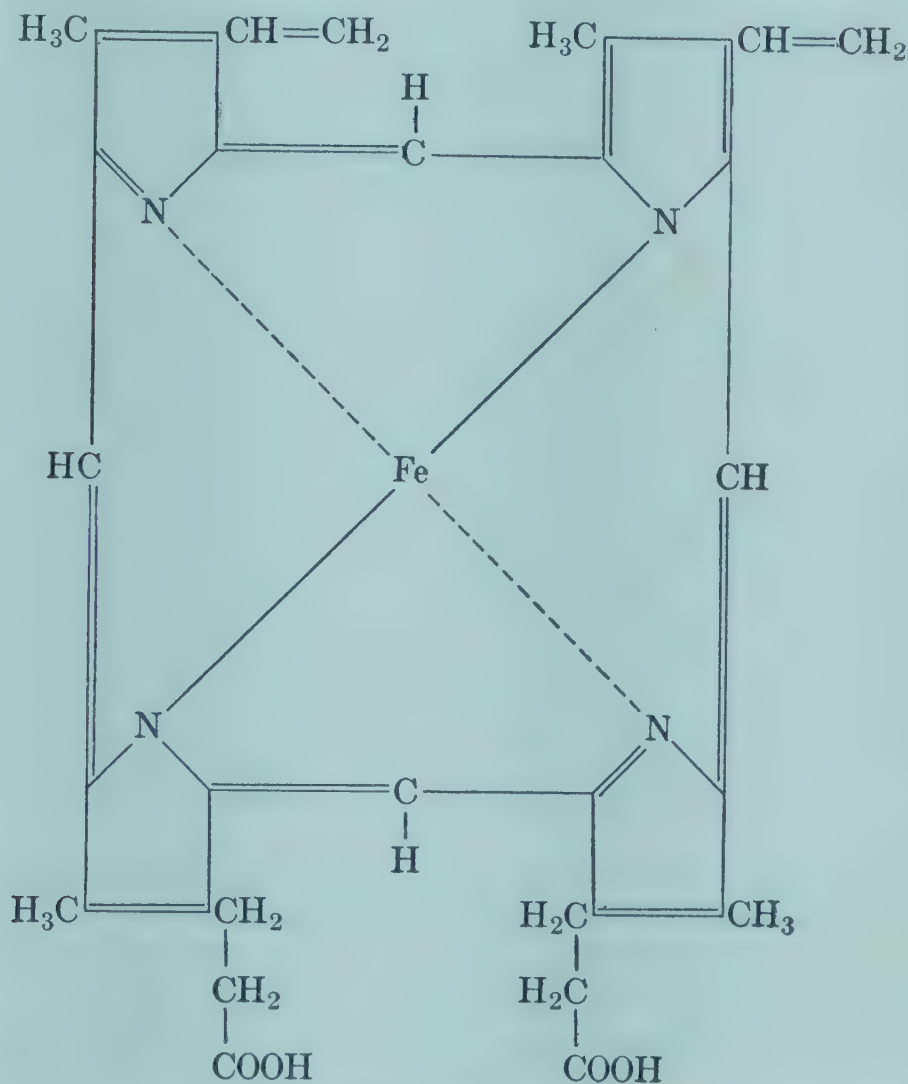
TABLE 9-V. NOMENCLATURE OF HEME AND ITS DERIVATIVES

Substance	Synonyms	Composition
Heme	Haem; ^a ferroheme; protoheme	Ferrous protoporphyrin IX
Hemin	Chlorohemin	Heme chloride
Hemoglobin	Ferrohemoglobin	4 Hemes + globin
Methemoglobin	Hemoglobin; ferrihemoglobin	4 Ferric protoporphyrins + globin
Myoglobin	Muscle hemoglobin	1 Heme + protein
Oxyhemoglobin		Ferrohemoglobin + O ₂
Carboxyhemoglobin		Ferrohemoglobin + CO
Hemichrome	Ferric hemochromogen	Ferric protoporphyrin IX + a base
Hemochrome	Ferrous hemochromogen	Heme + a base

^a In British usage the diphthong is used in all the words related to haemoglobin.

monia. These substances have been known as *hemochromogens*. It is now suggested that the two final syllables be dropped and that the valence of the iron be indicated, giving two types of compound, the *hemochromes* and the *hemichromes*. Table 9-V gives the names which are used for heme and its more important derivatives.

Structure of Hemoglobin. Heme as it occurs in hemoglobin is a ferrous derivative of protoporphyrin IX.



Heme
Ferrous protoporphyrin IX

Because of resonance this is a stable structure which has strong absorption bands in the visible region. This latter fact facilitated the early studies of hemoglobin and its derivatives, many of which were known only through their characteristic and easily observed absorption spectra. The molecule of heme is a flat disk, all the resonating atoms lying in the same plane with the iron atom at the center of this ring.

Of the six coördination positions available on the iron, four are used to bind the metal to the four nitrogens of the porphyrin. The two remain-

ing valences are directed, one upward and the other downward, at right angles to the plane of the ring. When heme is linked to globin it is believed that at least one of these free valences is involved.

Although it is no more possible to write a complete formula for globin than for any other protein, there is now available enough data to justify

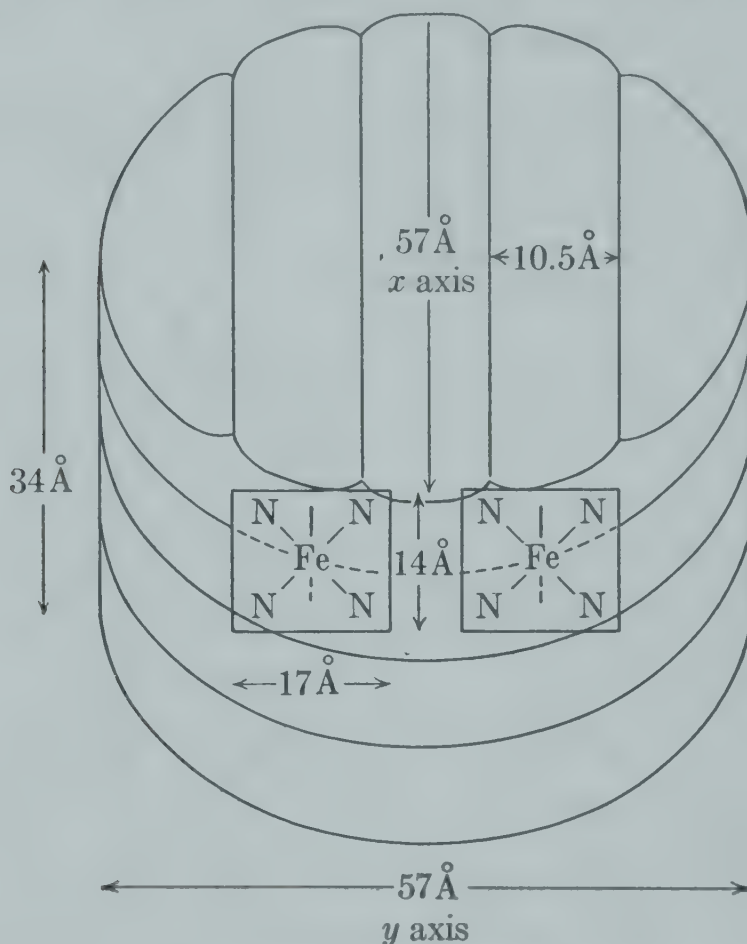
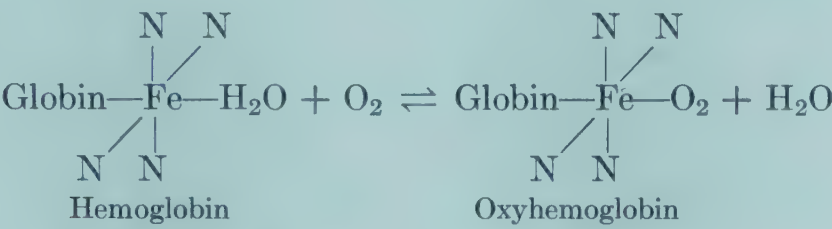


Figure 9.1. A molecule of horse hemoglobin as reconstructed from x-ray and biochemical data. In each of the four protein layers folded or coiled polypeptide chains make cylindrical rods with their long dimensions running along the x axis. Each layer has room for five such rods. The four heme molecules lie parallel to each other and perpendicular to the plane of the polypeptide layers. The two hemes which are not shown are attached to the other side of the protein, being bound to the two lower polypeptide layers. (From S. Granick, *The Harvey Lectures*, 44:220, 1948-49.)

a tentative description of this substance and of the complex which it forms with heme. This conjugation involves four heme residues per molecule of globin. X-ray studies of mammalian hemoglobin indicate that the protein is a cylinder, 34Å high with a diameter of 57Å . This cylinder does not have the form of a simple column, but is made up of four disks piled one upon the other as shown in Figure 9.1. Each disk in turn consists of folded polypeptide chains arranged to give a flat plate approximately 8.5Å thick. To this globin cylinder the four heme residues are attached. There is evidence that the heme groups, which are smaller disks 15Å in diameter and less than 4Å in thickness, lie in pairs parallel to each other

on the surface of the protein, perhaps as shown in the figure. Each heme is believed to be oriented and held in place by three bonds, two of them from the propionic acid groups of the heme to two properly placed basic groups in the protein, while another group in the globin is bound by one of the two free coördination valences of the iron atom. In this way each molecule of hemoglobin has available four free valences, one associated with each iron atom. When the molecule is oxygenated each of these valences holds an oxygen *molecule*. It has already been noted that oxyhemoglobin gives up its oxygen only in the presence of water. This is believed to depend on the need to keep the sixth coördination place filled, as can be done if the oxygen is simply displaced by a water molecule. These relationships are indicated below for a single heme residue, represented by its four nitrogens.



Life History of Hemoglobin in Man. The red corpuscles, or *erythrocytes*, of the adult are formed in the red bone marrow and after a life span which is about 120 days are destroyed. It has been estimated that approximately 10 million of these cells are normally removed from the circulation every second. This figure becomes less startling when it is realized that the total blood volume in the adult is between 5.5 and 6 liters and that every cubic millimeter contains approximately four million red cells.

When the red cells are destroyed the hemoglobin they contain is decomposed. This involves a daily loss of about 25 g. of pigment containing 85 mg. of iron. The daily excretion of iron however is only about 4.5 mg., indicating that the body conserves most of the iron and uses it again. As we shall see, the porphyrin part of the molecule is degraded and excreted, hence normal replacement of red cells involves a daily synthesis of about 1 g. of heme.

Synthesis of Heme: Until recently there was no way of knowing to what extent heme was synthesized in the body, except that a certain amount of iron was known to be essential to health. This situation changed when the problem was attacked by Shemin and Rittenberg⁵ and their colleagues

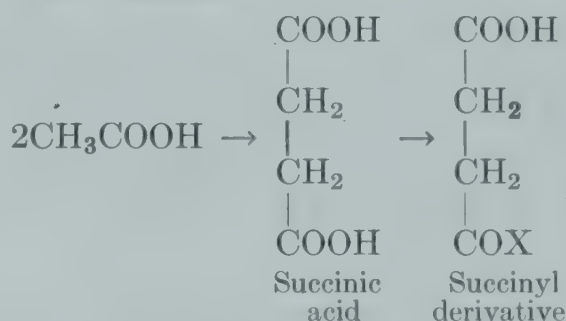
⁵ David Rittenberg (1906-) is now a Professor of Biochemistry at the College of Physicians and Surgeons of Columbia University. It was he who contributed to the original team of Schoenheimer and Rittenberg the specialized knowledge of isotopes which was as important as Schoenheimer's biochemical insight. His major interests lie in the fields of lipid and protein metabolism.

David Shemin (1911-) is an Associate Professor in the Biochemistry Department at the College of Physicians and Surgeons, and in work with Rittenberg is developing the use of isotopes to trace pathways of synthesis in the body.

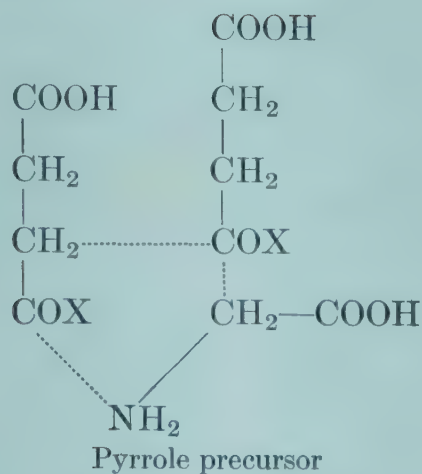
at Columbia University using isotopic labels. They showed first that in man ingestion of glycine ($\text{H}_2\text{N}-\text{CH}_2-\text{COOH}$) labeled with N^{15} was followed by a significant incorporation of the isotope in hemoglobin. In later experiments hemoglobin was synthesized *in vitro* by avian erythrocytes, which are nucleated cells. These confirmed the specific utilization of glycine not only to provide nitrogen for all four pyrrole rings, but to provide eight carbons, two per pyrrole, for the porphyrin ring. To determine whether both carbons of glycine were used, some of this compound was labeled in the α -carbon and some in the carboxyl carbon with C^{14} . When these were used separately as heme precursors the distribution of radioactivity in the product proved that it is only the α -carbon which is incorporated into the porphyrin. This means that synthesis of each pyrrole ring either uses several glycine molecules, or uses some other precursor in addition to glycine.

At least some of the remaining carbon atoms and one third of the hydrogen atoms of heme can be furnished by the methyl group of acetic acid which is of course present in tissue as a salt. This use of acetate was proved by experiments in which the two carbons of acetic acid were labeled separately with C^{14} , while the methyl group hydrogens were replaced by deuterium.

It is not yet possible to trace in exact detail the chemical reactions by which the porphyrin ring is built up, but certain facts have been established. It appears that an early step involves formation of the four-carbon succinic acid from two molecules of acetic acid, and that this is then transformed into a reactive succinyl derivative which has not yet been identified. The unknown group which renders this compound "active" is represented by X in the formula.



Two molecules of the reactive derivative are believed to condense with one of glycine to yield a substituted pyrrole which is the common precursor of all the pyrrole rings in heme. In the formulation the dotted lines indicate the linking of the three molecules to form the heterocyclic ring, but no attempt is made to define the small molecules which are split out. To form the large heme ring four of these precursors would be linked together, and the resulting compound would then undergo minor transformations of the side chains to yield the methyl and vinyl groups.



Destruction of Heme: After about four months of being buffeted about in the blood stream human red blood corpuscles are withdrawn from the circulation by special cells in the spleen and the liver. There they are broken up and digested and the hemoglobin is split into protein and the prosthetic group. The globin yields amino acids which become part of the metabolic supply of these substances to be used as are other split products of protein digestion. The iron is set free and carried back to the bone marrow to be incorporated in new heme molecules. But the porphyrin seems to be of no further use, for it undergoes chemical transformation in the liver and is excreted in the bile. This fluid is an external secretion of the liver which is formed continuously, stored in the gallbladder and released from time to time into the upper intestine. Some of the substances which it contains serve to facilitate the digestion and absorption of the lipids (see p. 325), but the *bile pigments* are simply excretory products derived from heme. These are, as the name indicates, colored substances which give the bile its yellowish-green color. As they travel down the gut they undergo chemical changes which darken them, making them chiefly responsible finally for the characteristic color of the feces.

The two chief bile pigments, bilirubin and biliverdin, are formed from heme by loss of iron and oxidative opening of the porphyrin ring at the α -methene group. This gives a product which is still a tetrapyrrole, with side chains arranged as in heme, but with its four pyrroles strung together in an open chain.

In Figure 9.2 is summarized the series of reactions by which the two bile pigments are believed to be formed from heme. The "IX α " following some of the names indicates that the arrangement of side chains is that of mesoporphyrin IX and that the break in the porphyrin ring has been made at the α -methene carbon.

As the bile pigments travel down the intestinal tract they are reduced, probably by intestinal bacteria, yielding among other products two orange-yellow compounds, urobilin and stercobilin. The relation of these compounds to bilirubin is outlined in Figure 9.3. It is probable that there

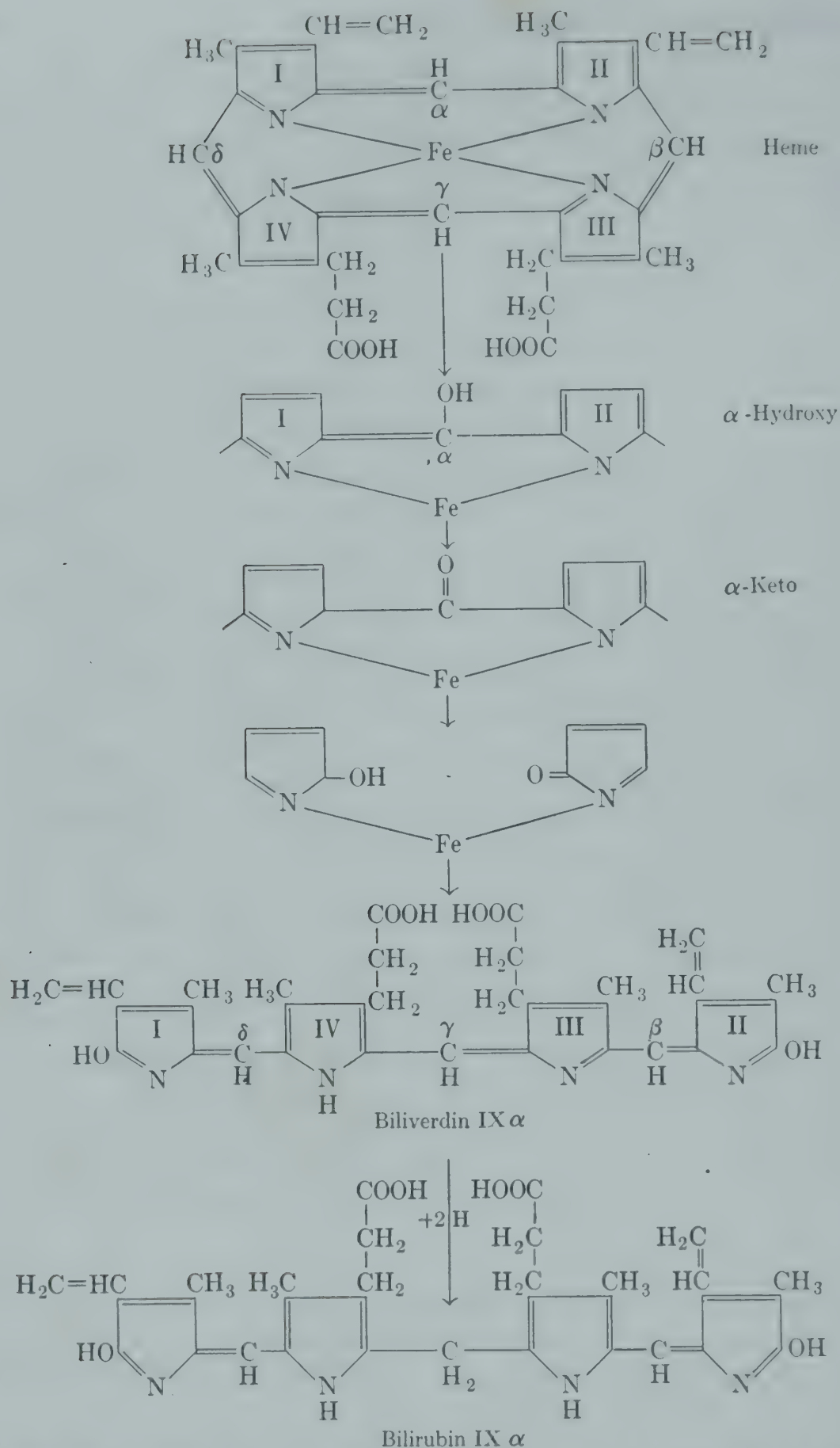


Figure 9.2. The conversion of heme to bile pigments. The opening of the large ring is believed to be an oxidative process. (From S. Granick and H. Gilder, "Distribution, Structure, and Properties of the Tetrapyrroles," in F. F. Nord [ed.], *Advances in Enzymology*, VII:336. Copyright 1947, Interscience Publishers, Inc., New York-London.)

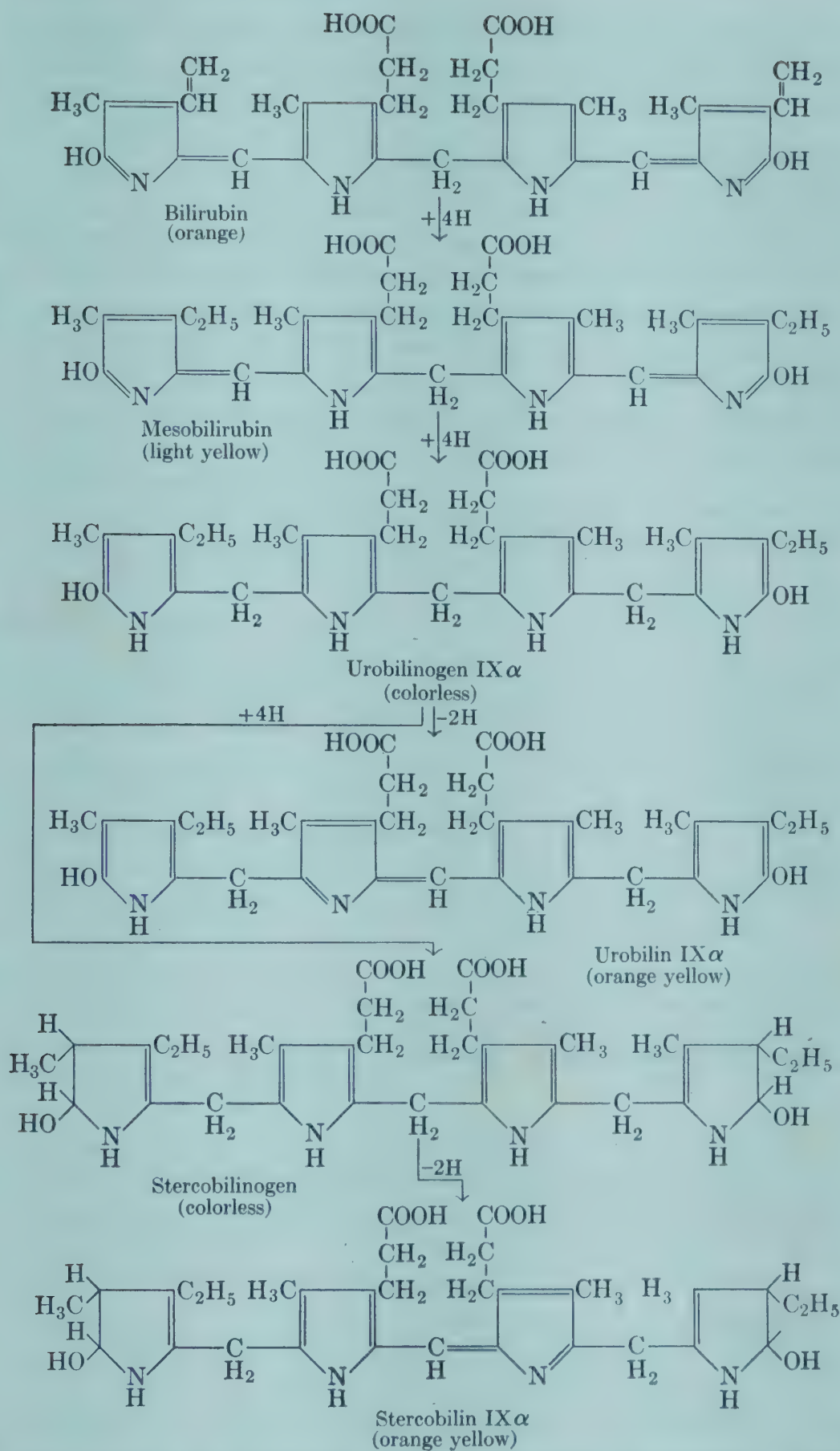


Figure 9.3. Stages in the transformation of bilirubin to urobilin and stercobilin. (From C. J. Watson, *The Harvey Lectures*, 44:44, 1948-49.)

are still other final products of a deeper color, since these two do not adequately account for the actual color of feces. But so far the other pigments which occur in feces have not been characterized chemically.

TRANSPORTATION OF THE BLOOD GASES

Hemoglobin is involved not only in oxygen transport but in the ability of the blood to carry carbon dioxide without appreciable disturbance of its pH. Although these functions are exercised in much the same way in all bloods which contain hemoglobin, it should be noted that the quantitative data used in the following discussion have nearly all been obtained with human blood.

Transportation of Oxygen. The affinity of hemoglobin for oxygen is such that in the short time required for blood to traverse the capillaries of the

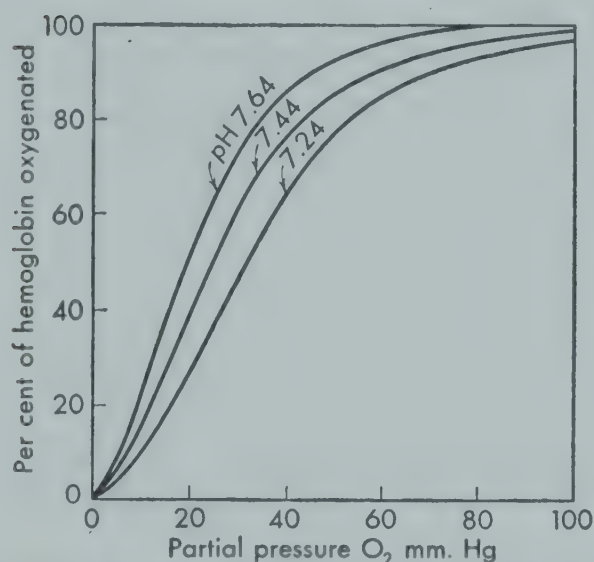


Figure 9.4. Effect of oxygen pressure and of acidity on oxygen dissociation curve of blood. (From J. F. Fulton [ed.], *Textbook of Physiology*, Saunders, Philadelphia, 1949. After Peters and Van Slyke.)

alveoli the pigment is very nearly saturated with oxygen, while at the oxygen tension which obtains in the tissues nearly half of this oxygen is released. The partial pressure of oxygen in the alveoli is approximately 100 mm. and at this pressure each 100 ml. of blood holds about 19.6 ml. of oxygen. In the tissues the oxygen tension is 35 mm. or less and as a result 7 to 9 ml. of oxygen is set free by each 100 ml. of blood traversing the capillaries.

The quantitative relationship between oxygen pressure and the dissociation of oxyhemoglobin is shown in Figure 9.4 in which the oxygenation of whole blood at three different pH values is plotted against the oxygen pressure. The general shape of the curves indicates that the relation is not a simple proportionality which would give a straight line, while the differences between the three show that the extent of dissociation of oxyhemoglobin is also a function of the pH. Thus at a partial pressure of 40 mm. and pH 7.24 a significantly larger volume of oxygen is set free than at a pH of 7.44. This is of importance because of other changes taking place in the blood as it passes through the tissues. The release of oxygen to the tissues is accompanied by a transfer of carbon dioxide from the tissues to the blood stream and this tends to lower the pH slightly just when a lowered pH is advantageous. On the other hand oxyhemoglobin is a stronger acid than hemoglobin, the pK_1 value for horse hemoglobin being 6.68 for

oxyhemoglobin and 7.93 for hemoglobin. This means that the tendency of the entering carbon dioxide to lower the pH of the blood is counterbalanced in part by the transformation of an acidic compound into a weaker acid. The fact that the curves are steepest between 20 and 40 mm. pressure makes sure that adequate amounts of oxygen will be made available to the tissues at their normal levels of oxygen tension.

Carbon Dioxide Transport. A small amount of carbon dioxide is carried in the blood in true solution but the major part is present as bicarbonate ion with smaller amounts as carbonic acid and perhaps 10 per cent as a hemoglobin complex known as a *carbamino compound*. Although this compound is frequently indicated by the formula $HHbCO_2$ this does not mean that the carbon dioxide replaces oxygen and is attached to the iron. It is believed that carbon dioxide reacts directly with free amino groups in the globin to form carboxyl groups:



All the various forms in which carbon dioxide is carried are in equilibrium with each other and when reference is made to the "volume" of carbon

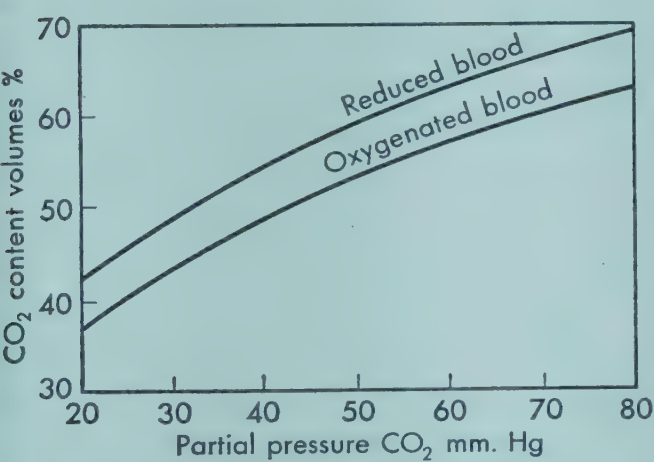


Figure 9.5. Carbon dioxide content of arterial and venous blood at various carbon dioxide pressures. (From J. F. Fulton [ed.], *Textbook of Physiology*, Saunders, Philadelphia, 1949. After Peters and Van Slyke.)

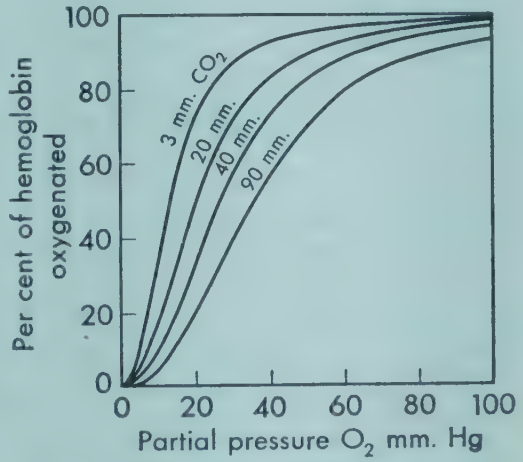


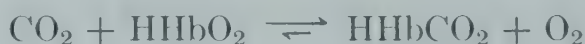
Figure 9.6. The effect of different concentrations of carbon dioxide on the dissociation of oxyhemoglobin. (From J. F. Fulton [ed.], *Textbook of Physiology*, Saunders, Philadelphia, 1949. After Barcroft.)

dioxide in blood it is to be understood that this refers to the sum of all these forms, reported in terms of the volume of gas which would be used in forming them, or which can be released by suitable procedures.

As might be expected, the volume of carbon dioxide absorbed by the blood depends in part on the partial pressure of the gas itself. This is illustrated in Figure 9.5 in which the volume of carbon dioxide which can be taken up by arterial and by venous blood is plotted against the carbon

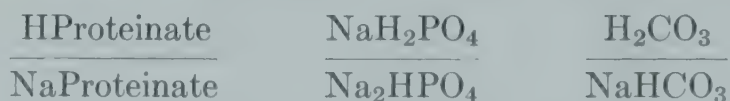
dioxide tension. The fact that the two curves are different indicates that carbon dioxide uptake depends also upon the partial pressure of oxygen in the system. Thus arterial blood, in which the oxygen concentration is high, carries less carbon dioxide at a given carbon dioxide tension than does venous blood with its lower oxygen content. Another facet of this relationship is brought out in Figure 9.6 in which can be seen the effect of different partial pressures of carbon dioxide on the oxygenation of whole blood. A result of this influence is that a high carbon dioxide tension facilitates release of oxygen just as a high partial pressure of oxygen hastens the evolution of carbon dioxide.

A small part of the carbon dioxide which enters the blood stream reacts slowly with water in the plasma to form carbonic acid or bicarbonate ion. But the major part of it diffuses into the red cells where there are two special mechanisms for dealing with it. Inside the red cell but not in the plasma is an enzyme, *carbonic anhydrase*, which acts to speed up the normally rather sluggish hydration of carbon dioxide to carbonic acid. At the same time some of the carbon dioxide which enters the red blood cells reacts there with oxyhemoglobin to form the carbamino compound.



This hastens the release of oxygen, for the carbamino compound has a smaller affinity for oxygen than has hemoglobin itself. As indicated, the reaction is reversible and when the blood reaches the lungs where the oxygen tension is high and the pressure of carbon dioxide is relatively low the reverse reaction facilitates the expulsion of carbon dioxide and oxygenation of hemoglobin.

The Blood Buffers. It has been noted many times that in spite of the reactions which have just been outlined the pH of the blood is held remarkably constant at about 7.40. This is achieved partly by the change in the strength of the hemoglobin acids, the weaker hemoglobin appearing simultaneously with the influx of acidic carbon dioxide, and the stronger oxyhemoglobin at the time when carbon dioxide is leaving the blood. But the main defense against changes in acidity is the series of buffer pairs listed below:



Of these the proteins, and especially the two hemoglobin pairs, those of reduced and of oxyhemoglobin, are quantitatively most important.

Examination of the titration curves for the inorganic buffer pairs, the carbonates and orthophosphates (Fig. 9.7), shows that at the pH of blood neither pair is present in optimum buffering ratio. At pH 7.4 the ratio of NaH_2PO_4 to Na_2HPO_4 is about 1:4, which does not give a wide buffering

capacity but does provide effective resistance to a small increase in acidity. Since normal metabolic changes are more likely to give rise to acidic than to basic products this is a useful provision.

At the same pH, the ratio of carbonic acid to its salt seems even less effective, being approximately 1:20. In spite of this low ratio, the carbonate pair constitutes a good physiological buffer because respiratory removal of carbon dioxide keeps this ratio nearly constant. When acid enters the blood it is neutralized by the bicarbonate present and such a

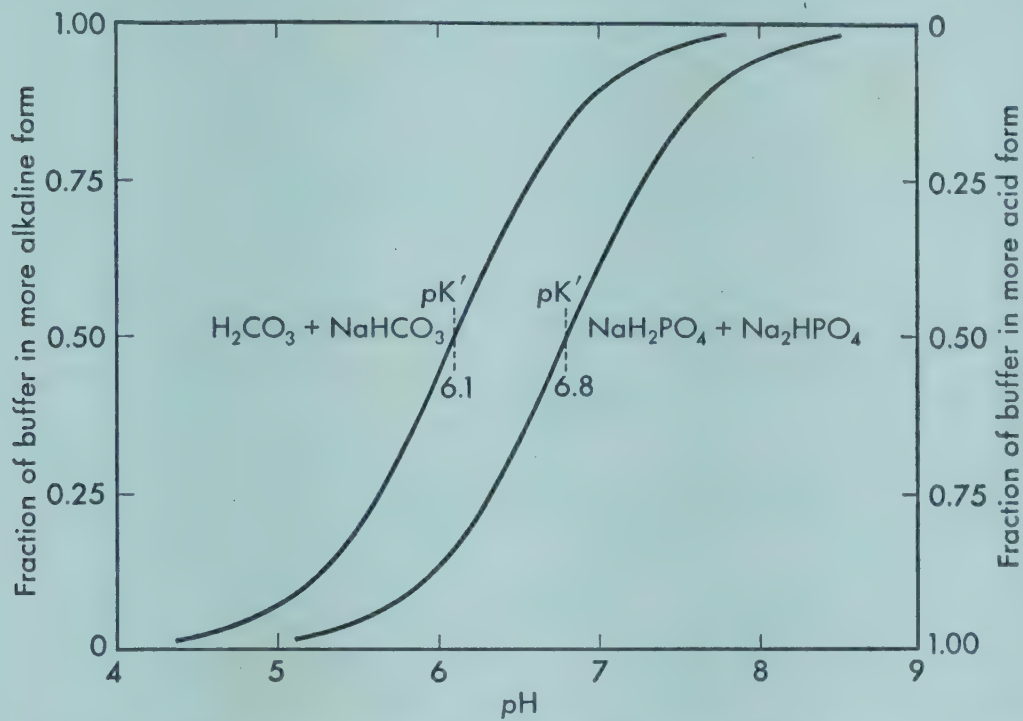
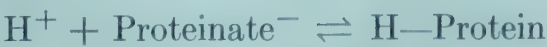


Figure 9.7. Dissociation curves of the blood buffer acids, H_2CO_3 and H_2PO_4^- . (From J. F. Fulton [ed.], *Textbook of Physiology*, Saunders, Philadelphia, 1949.)

reaction in a solution would change the buffer ratio by decreasing the salt concentration and increasing that of the acid. But in the body the extra carbon dioxide is eliminated promptly and as long as the bicarbonate is not entirely exhausted a buffer ratio close to the normal 1:20 is maintained. Because of its function in neutralizing acids the bicarbonate in the blood has been called the “alkaline reserve” of the body. Actually the total alkaline reserve consists of bicarbonate ion, secondary phosphate ion, $\text{HPO}_4^{=}$, and all the anionic proteins which can accept hydrogen ion and go over into unionized form.



As a result of all the adjustments which take place when acidic substances enter the blood stream the pH of venous blood is only about 0.02 pH unit lower than that of arterial blood.

Summary. Figures 9.8 and 9.9 indicate diagrammatically the important events which take place as blood traverses the capillaries of the alveoli

and the capillaries of the tissues. It should be remembered that although the reactions are represented with single arrows they are all reversible.

Blood reaches the alveolar capillaries carrying carbon dioxide as bicarbonate ion in cells and plasma and as hemoglobin carbamino compound inside the erythrocytes. It should be noted that even in very active tissues where the need for oxygen is urgent, only part of the oxyhemoglobin

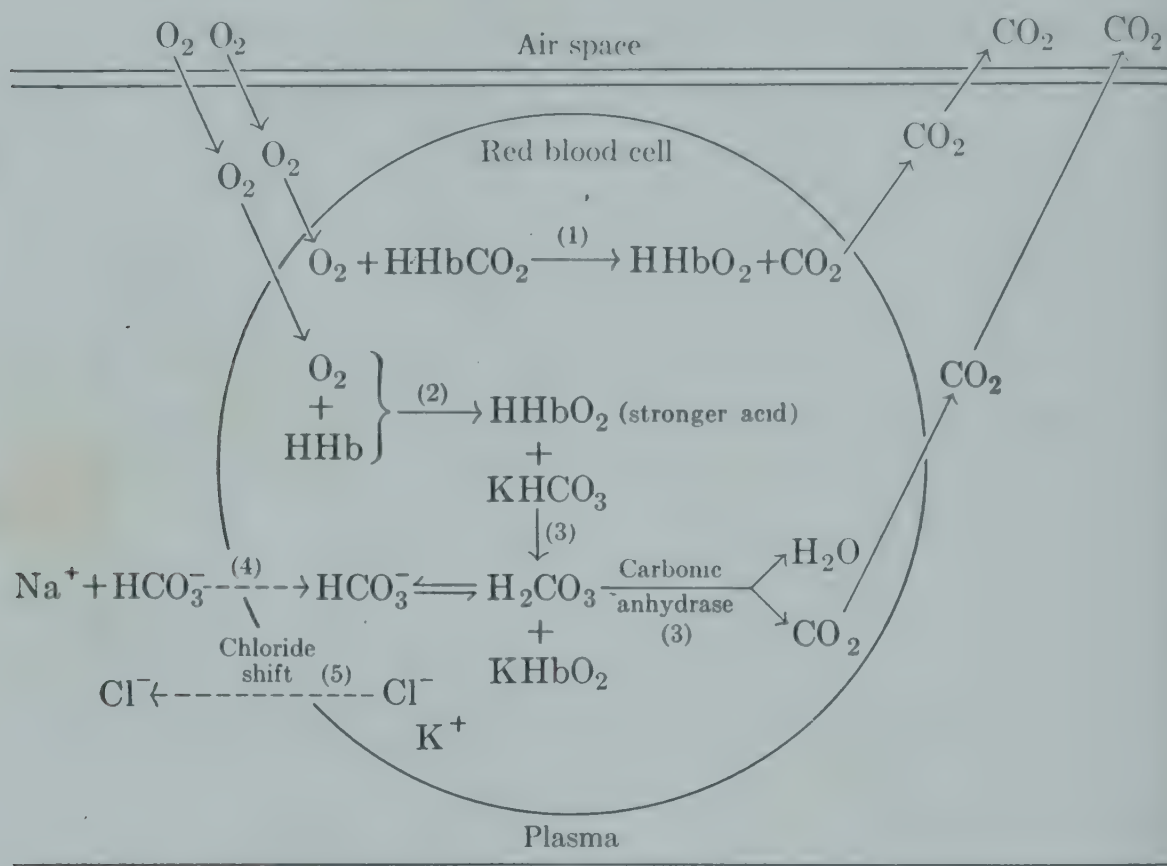


Figure 9.8. Diagrammatic representation of the chemical changes which take place in the capillaries as the blood moves through the lining of the alveoli. The passage of oxygen into the blood is a result of the high oxygen tension in the alveoli, while the carbon dioxide is driven out of the blood by its higher pressure there. (From J. F. Fulton [ed.], *Textbook of Physiology*, Saunders, Philadelphia, 1949.)

dissociates in the tissues, and so the cells of the venous blood always contain both reduced and oxygenated hemoglobin. As the blood flows through the capillaries of the alveoli, oxygen diffuses across the capillary wall into the plasma and thence into the erythrocytes. The chemical changes which take place may be summarized as follows:

1. Oxygen reacts with the carbamino compound, releasing its carbon dioxide.
2. Oxygenation of the reduced hemoglobin gives rise to an acid, $HHbO_2$, which is a stronger acid than HHb .
3. This increased acidity is neutralized by the $KHCO_3$ in the cell, thus freeing more carbon dioxide. The rate of this reaction is high because

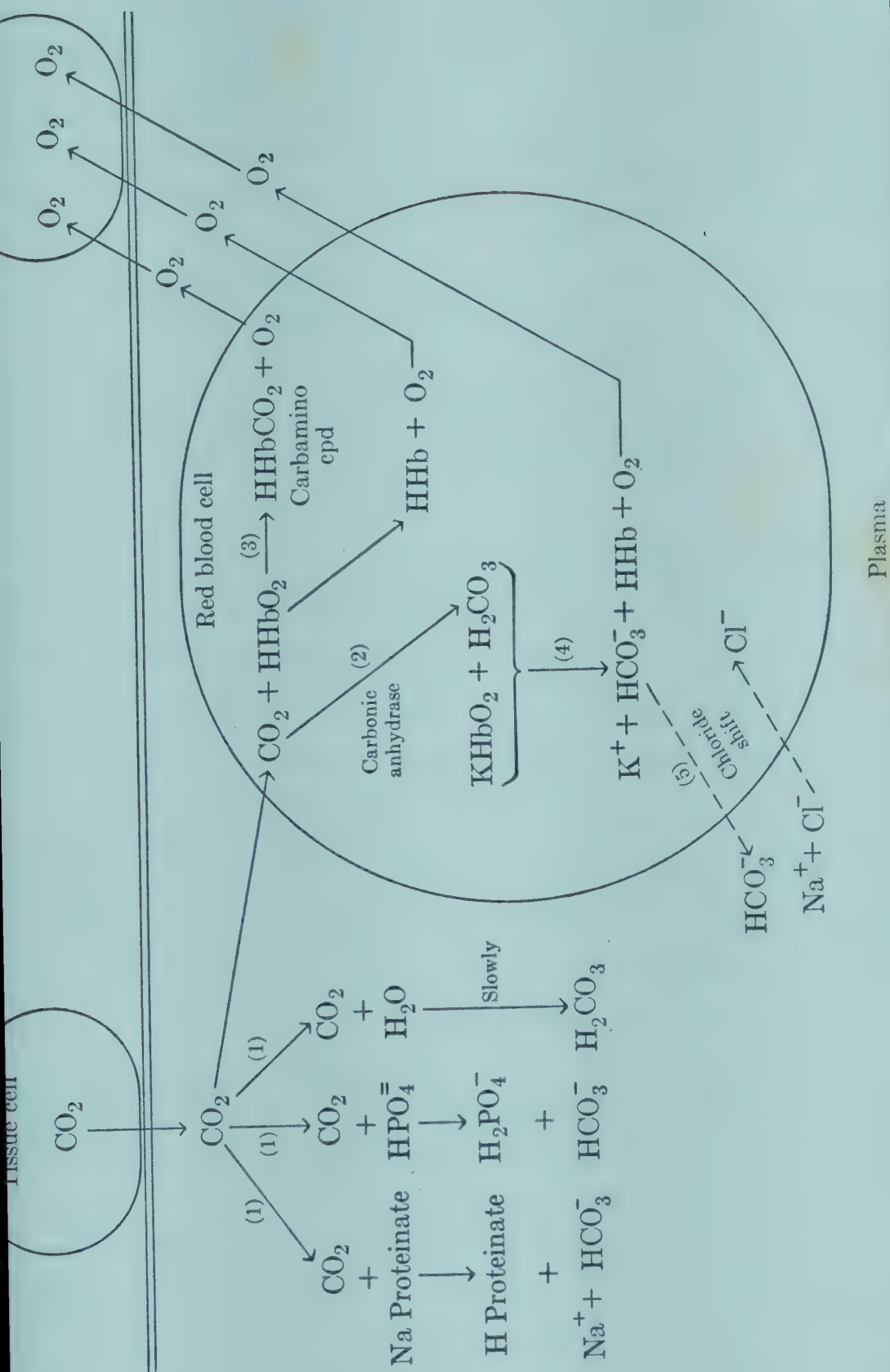


Figure 9.9. Diagrammatic representation of the chemical changes which take place as the blood moves through the capillaries of the tissues. Here the carbon dioxide tension of the tissue cells is higher than that of the plasma and the oxygen tension is lower. This leads to a movement of carbon dioxide into the blood and a movement of oxygen into the cells.

of the presence in the cells of carbonic anhydrase which greatly hastens the decomposition of carbonic acid as it forms.

4. Escape of carbon dioxide lowers the concentration of bicarbonate ion in equilibrium with carbonic acid inside the cells and this allows more bicarbonate ion to move into the cells from the plasma.
5. The increased concentration of bicarbonate ion in the cells is balanced by a simultaneous migration of chloride ions out into the plasma. This reciprocal change in the ratio of chloride ion to bicarbonate ion in cells and plasma is known as the *chloride shift*.

As a result of this series of reactions blood leaves the lungs with a diminished load of carbon dioxide and carrying about 95 per cent of its hemoglobin in the oxygenated form.

In the tissues the oxygen tension may be as low as 30 mm. and the pressure of carbon dioxide is 50 mm. or more. The reactions which ensue when the oxygenated blood reaches the tissue capillaries are as follows:

1. Carbon dioxide moves into the plasma and there reacts slowly with water and with buffers, including the plasma proteins, to form bicarbonate ion and a small amount of carbonic acid.
2. Most of the carbon dioxide enters the cells and there reacts rapidly with water, under the influence of carbonic anhydrase.
3. Another part of the carbon dioxide which diffuses into the cells reacts with oxyhemoglobin to form carbamino compound and to release oxygen to the tissues.
4. Part of the oxyhemoglobin dissociates, its oxygen diffuses away, and the hemoglobin which remains is a weaker acid than was the oxygenated acid. This means in effect that the oxyhemoglobin in giving up its oxygen is enabled to accept part of the hydrogen ion formed in Step 2, when carbon dioxide reacts with water.



5. The increased concentration of bicarbonate ion in the cells drives it into the plasma and this in turn causes chloride ions to invade the cells. Thus in the tissues there is a chloride shift which is the reverse of that which occurs in the lungs.

Leaving the tissue capillaries the blood returns to the heart whence it is sent to the lungs to dispose of its carbon dioxide and to be recharged with oxygen.

RESPIRATORY PIGMENTS

In the preceding discussion of the respiratory functions of human blood, the word hemoglobin has been used as if it referred to a definite chemical

entity. Actually many different "hemoglobins" occur, not only in the vertebrates but in some species of invertebrates, and even in at least one place in the plant world (see p. 295). These compounds are called by a single name because they are all conjugated proteins which react reversibly with oxygen and have heme as prosthetic group. But they differ among themselves in physical and chemical properties, including solubility, crystalline form, absorption spectra, and quantitative response to changing oxygen tension. Corresponding with these differences it has been found that the protein parts of the various hemoglobins differ widely in molecular weights and in amino acid composition.

The hemoglobins of vertebrate bloods, while they show some species differences, have a uniform molecular weight of approximately 67,000, and consist of a globin united with four heme residues. But the hemoglobin of vertebrate muscles, myoglobin, with a molecular weight of only 17,000 and but one heme group per molecule, seems to be one fourth of a blood hemoglobin molecule. Contrasted with both of these are the invertebrate hemoglobins, some of which contain over 100 heme groups and have been isolated with molecular weights as high as 3,000,000. These very high molecular weights are usually found in organisms in which the hemoglobin is in solution in the plasma instead of being confined inside a cell membrane. This may reflect the need to provide a good many heme groups for oxygen transport without increasing too greatly the osmotic pressure of the plasma.

Another property which varies greatly from one hemoglobin to another is the affinity for oxygen, usually measured in terms of the oxygen tension required to cause half saturation. This depends, as we have seen, upon the *pH* and also upon the temperature, but when these are allowed for it is found that some hemoglobins hold oxygen so tenaciously that they do not release it even to a vacuum, while others give it up at relatively high partial pressures of oxygen. In most cases these differences prove to reflect the differing conditions under which the pigment must function. We have already seen how well human hemoglobin is adapted to the conditions it meets in human tissues. In the same way, organisms which live where the oxygen tension is low are equipped with a hemoglobin which can be saturated at low pressures. Others which normally obtain their oxygen by simple diffusion carry in their body fluids a variety of hemoglobin which does not give up its oxygen until the oxygen tension in their environment has fallen too low to meet their needs by diffusion. Then the reserve of oxygen in hemoglobin is released to carry them past the time of crisis. In Table 9-VI the affinities of several different hemoglobins for oxygen are listed in terms of oxygen tensions required for half saturation. Obviously the lower the pressure recorded the greater the oxygen affinity of the hemoglobin.

TABLE 9-VI. OXYGEN AFFINITIES OF VARIOUS HEMOGLOBINS

Hemoglobins	Temp. (°C.)	Oxygen Tension for Half Saturation
Human	35	7.5 mm.
Frog	35	42
Human	15	0.3
<i>Planorbis</i> (snail)	15	2
<i>Chironomus riparius</i> (midge)	17	0.6
<i>Daphnia magna</i> (water flea)	17	3.1
Root nodules of leguminous plants	15	>0.1
Nematode (round worm)	19	>0.1

Besides the hemoglobins there are at least three other animal pigments or groups of pigments which are used in oxygen transport. These are the *chlorocruorins*, the *hemocyanins*, and the *hemerythrins*.

Chlorocruorin is a dichroic substance, red in concentrated and green in dilute solution. It is found in solution in the plasma of certain marine worms, and is closely related to hemoglobin. Its prosthetic group is a ferrous tetrapyrrole which differs from heme only in having an aldehyde group in place of the vinyl group at position 2. But the protein part of the pigment is very different from globin since the molecular weight of chlorocruorin as isolated is in the neighborhood of 2,500,000. It differs from hemoglobin also in having a much lower affinity for oxygen than have most hemoglobins.

The other two pigments have been less completely characterized. Hemocyanin, which is blue in the oxidized form, has an apparently random distribution in the blood of some molluscs and arthropods. The prosthetic group, which can be split from it by treatment with alkali, contains copper rather than iron. Its structure is not known though it seems to be partly polypeptide in nature. When the pigment reacts with oxygen it is in a ratio of two atoms of copper to each molecule of the gas. The molecular weights of the hemocyanins are very high, and this again is to be correlated with their occurrence in solution in the body fluids rather than in cells.

Hemerythrin is a rare pigment which occurs only in the cells of sipuncu-
lid worms. Almost nothing is known of its chemistry except that its prosthetic group is not a porphyrin though it does contain iron. The molecular weight of the compound is about 67,000 of which just over 1 per cent is iron, which means that it contains three times as much iron per molecule as does hemoglobin. Since, however, it unites with oxygen in the ratio of three atoms of iron to one molecule of oxygen, each molecule carries the same amount of oxygen as does a molecule of hemoglobin. The known properties of the respiratory pigments are collected in Table 9-VII.

TABLE 9-VII. RESPIRATORY PIGMENTS ^a

Source	Hemoglobins		Chlorocruorin	Hemocyanin	Hemerythrin
	Vertebrates	Invertebrates ^b	Marine Poly- chaete Worms	Arthropods and Molluscs	Sipunculid Worms
Mol. wt. range ^c	67,000	17,000– 3,400,000	2,750,000	400,000– 10,000,000	66,000
Metal	Fe—0.34%		Fe—1.20%	Cu—1.25%	Fe—1.01%
Prosthetic group	Heme		Ferrous protoporphyrin	Polypeptide ?	?
Molecules O ₂ per atom metal	1:1		1:1	1:2	1:3
Occurrence	Cells or plasma		Plasma	Plasma	Cells
Colors	Red		Green ⇌ Red	Blue	Red

^a Most of the data from T. Svedberg, *Biol. Bull.*, 71:489, 1936.
^b The invertebrate hemoglobins have also been called erythrocruorins.
^c Molecular weights are given in round numbers.

THE HEME ENZYMES

Besides the hemoglobins there is another group of hemoproteins which occurs widely distributed in plant and animal tissues. These are the compounds in which heme or some closely related heme derivative occurs as part of the oxidizing enzymes catalase, peroxidase and the cytochromes. Of these catalase and the cytochromes are found in nearly all aerobic cells; peroxidase is particularly characteristic of plants although it is also found in some animal material. The hemoprotein enzymes differ from the hemoglobins both in the very specific protein components and in the oxidation state in which iron occurs. In catalase and peroxidase as isolated, the iron is in the ferric state and it is still a matter of argument whether or not the catalytic action involves changes in the valence of the metal. The cytochromes are known to act through alternating oxidation and reduction of iron. There is a further discussion of the hemoprotein enzymes in the chapter on Biological Oxidations.

Transport in Plants

Although the transportation systems of plants are apparently less complex than those of animals, much less is known of the actual mechanism by which substances move from one area of the plant to another. Multicellular plants like multicellular animals have to make provision for many cells which are not in immediate contact with essential nutrients or raw materials. Thus the roots, while they do have effective contact with the salt solutions of the soil, must be furnished with organic foodstuffs. The leaf cells on the other hand synthesize their own organic foods from carbon dioxide and water, but in order to build such compounds as phospholipids and proteins must acquire from the soil the nitrogen, phosphorus, and sulfur which occur there in inorganic ions.

ABSORPTION OF WATER

The root hairs, each of which consists of a single surface cell with a fine, threadlike extension, are formed just behind growing root apices and usually occupy only a few centimeters of root. As the root grows through the soil, the old hairs die and are replaced by others nearer the tip, so that the root progressively draws upon new layers of soil. Here the root hairs are in close contact with the soil water which holds in solution not only inorganic ions but also some oxygen and perhaps small amounts of carbon dioxide in the form of carbonates. The solute concentration in ordinary agricultural soils is low, so that the osmotic pressure is usually less than an atmosphere. In the root hairs on the other hand the osmotic pressure may be 5 atmospheres or more. This osmotic gradient is undoubtedly one factor in the movement of solution into the root cells.

There is abundant evidence however that the process is not simply a passive response to osmotic differences. For example, when carrot root tissue is immersed in isosmotic solutions of sucrose, calcium chloride, and potassium sulfate, considerably less water is absorbed from the calcium chloride solution than from the others. This has been interpreted as evidence that the entrance of water is controlled to some extent by the distribution of electric charges at the surface of the cell. Inequalities of distribution might arise through different mobilities of diffusing cations and anions, or through selective adsorption of anion or cation on the membrane. To the extent that diffusion of water is dependent upon charge distribution it is said to represent *electrosmotic absorption*.

Finally there is experimental evidence that water absorption is also in some way dependent upon the metabolic reactions of the living cell, for absorption is reduced when the respiration of root cells is curtailed by poor aeration of the soil. Thus the process appears to be not merely a passive response to the laws of osmosis, but an active one in the sense that the energy of respiration is utilized in some way to draw water into the cells. At present there is no indication of the way in which oxidation and water absorption are linked.

ASCENT OF WATER AND SOLUTES

As a result, then, of factors which still need to be clarified, a dilute aqueous solution enters the root hairs from the soil, and passes through the living root cells into the xylem tissue. This is made up predominantly of dead cells, some of which are joined end to end to form the long vessels known as *tracheae*. Other cells, the *tracheids*, have pitted walls through which water passes from cell to cell. If a well-watered plant is cut off just above the soil, liquid often exudes for some time from the cut surface of the stump, showing that a pressure has developed which tends to force water from the root cells into the xylem. This is known

as *root pressure*, and is believed to result from the osmotic gradient between the root cells and the more concentrated solution in the xylem cells. How the higher osmotic pressure is maintained in the dead tracheae and tracheids is not known, but it has been suggested that there is a continuous diffusion of sugar into these regions from the living cells which border them.

The aqueous solution which enters the xylem in the roots is carried upward to the leaves where the salts are used and most of the water evaporates. This *transpiration stream* may rise several hundred feet, moving at a rate which is too rapid to be entirely dependent on osmosis, and reaching heights too great to be accounted for by capillarity or by root pressure. In the course of the years many theories have been advanced to account for this phenomenon, but it is still very imperfectly understood. It is generally agreed that the ascent depends in part at least on the tensile strength of the water itself. It has been shown that at least 150 atmospheres pressure must be exerted to break the cohesion between water molecules, and from this it was reasoned that continuous columns of liquid in the xylem would behave to some extent like solid rods. As water evaporates from the leaf surface, the salt concentration of the remaining solution increases. The resulting elevation of osmotic pressure in the leaf cells tends then to draw water out of the upper ends of the xylem columns. Thus to the feeble upward root pressure is added a pull on the water from above. This upward pull, being far too small to break the cohesion of the water, is transmitted to the column which is thus drawn upward as a whole.

Although the cohesion of water is doubtless an important factor in the normal transport of water and salts from the soil to the leaves, the process is undoubtedly far more complex than this simple theory would indicate. For one thing, it has been found that plants can be maintained in an atmosphere saturated with water vapor. Under these conditions transpiration is reduced to zero, and yet the salts from the soil rise to the leaves, for photosynthesis goes on and the plant continues to grow. Thus it is clear that this movement of essential raw materials into the leaves can be brought about even without the usual upward movement of a water column.

TRANSPORT OF PHOTOSYNTHETIC PRODUCTS

Of the compounds formed during photosynthesis only a small fraction is needed to maintain the leaf tissue itself. The greater part of this material is translocated either to actively growing parts of the plant where it is used immediately in building or nourishing tissue, or to places where it is stored for future use. This movement of nutrients away from the leaves takes place through the phloem. Here the actual conducting tissue consists largely of *sieve cells* which, with perforated end walls, form a con-

tinuous conducting system from the living cells of the leaves to the roots, to the apices of growing shoots, and to the varied places of storage in the plant. Through the phloem are transported not only soluble sugars but probably various other small molecules such as amino acids and organic phosphates. These compounds may be oxidized or they may be used synthetically by the cells to which they are carried. If they are to be stored they are transformed into large or otherwise insoluble molecules and stored as starch or protein or fat.

As with the movement of material in the xylem elements of the plant, there is no generally accepted explanation of the mechanism of translocation in the phloem. The rate at which sucrose, for example, travels through the phloem is many thousand times the rate at which it diffuses through water. Various theoretical mechanisms have been suggested to account for this, but none is entirely adequate.

In plants other than annuals the process of translocation just outlined is reversed in the spring. Starch or protein reserves are hydrolyzed to yield small soluble molecules and these move, again through the phloem cells, but now often in the opposite direction, to the growing parts of the plant. Here they furnish substrates for oxidation and for synthesis until photosynthesis begins.

There is in plants no need for an elaborate system of oxygen transport, for the energy requirements of most plant cells are low compared with the requirements of many animal cells. A large part of the living tissue of the plant consists of thin, porous leaves the cells of which are in direct contact with air through the stomata, and the oxygen needs of other cells are adequately met by the small amounts of oxygen dissolved in soil water, or by simple gas diffusion into the intercellular spaces.

Suggestions for Further Reading

GENERAL

FULTON, J. F. (ed.), *A Textbook of Physiology*, Saunders, Philadelphia, 1949.

This book includes very full treatment of both the chemistry and physiology of the blood.

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CORWIN, A. H., "The Chemistry of the Porphyrins," in Gilman, H. (ed.), *Organic Chemistry, An Advanced Treatise*, 2nd ed., Wiley, New York, 1943.

- FOX, H. M., "On Chlorocruorin and Haemoglobin," *Proc. Roy. Soc., B* 136:378, 1949-1950.
- GRANICK, S., "The Structural and Functional Relationships between Heme and Chlorophyll," *The Harvey Lectures*, 44:220, 1948-1949.
- GRANICK, S., and GILDER, H., "Distribution, Structure and Properties of the Tetrapyrroles," *Adv. in Enzym.*, 7:306, 1947.
- KEILIN, D., and WANG, Y. L., "Haemoglobin in the Root Nodules of Leguminous Plants," *Nature*, 155:227, 1945.
- WYMAN, J., JR., "Heme Proteins," *Adv. in Prot. Chem.*, 4:410, 1948.

Study Questions

1. List the functions of the blood.
2. What is the difference between plasma and serum?
3. What ions occur in unequal concentrations in blood cells and serum?
4. Write the equation for the fundamental reversible reaction of hemoglobin with oxygen. What factor determines the equilibrium point for this reaction?
5. What is heme? hemin? the structure of pyrrole?
6. What is the porphyrin structure? What is meant by saying that heme exhibits resonance? What is the general shape of the heme molecule?
7. Where in the plant world has hemoglobin been found?
8. Name three enzymes which contain heme or some closely related compound.
9. Name several blood buffer pairs. Explain how the difference in the ionization constants of hemoglobin and oxyhemoglobin helps to maintain a constant pH in the blood.
10. Why is the bicarbonate buffer pair effective in spite of the fact that the ratio of acid to salt is 1:20? Why would such a ratio be ineffective in an isolated solution?
11. What factors are believed to be involved in the passage of soil water into the root hairs?
12. Through what structural element does soil water move from the roots to the leaves?
13. What is "root pressure" and what experimental evidence is there that it exists?
14. State one experimental fact which indicates the inadequacy of the cohesion theory to explain the movement of water from soil to leaves?
15. What is meant by "electrosmosis," and what evidence is there that such a factor plays a part in the passage of solutes into the roots?
16. In what forms are photosynthetic products transported? In what forms are they stored?
17. Through what tissue is the stored material in a perennial plant translocated when it is to be used? In what form?
18. How are the living root cells supplied with oxygen?

Digestion and Absorption

The fundamental problem of biology, viewed as the science of living things, is undoubtedly that of assimilation and growth.

W. P. D. WIGHTMAN: *The Growth of Scientific Ideas* (1951)

With the possible exception of a few pampered bacteria, living in luxury in laboratory test tubes, nearly all living organisms must carry on digestive processes of greater or less complexity. This is enforced by the fact that most foodstuffs are ingested or stored in the form of large, insoluble molecules. Proteins and polysaccharides must be hydrolyzed to simpler compounds in order that they may pass through cell membranes for transportation and use. Even the comparatively simple fats are not absorbed until at least part of them have been hydrolyzed. Furthermore, although the heterotrophic organisms require some degree of complexity in their foodstuffs, the prescribed substrates for cellular oxidation and synthesis are not proteins and polysaccharides but simple monosaccharides, amino acids, fatty acids, and similar small molecules. Therefore for the most part the digested foodstuffs are absorbed and transported in these forms.

In plants the digestive demands are relatively simple. Yet plants elaborate and presumably use many enzymes which are entirely analogous to the animal proteases, carbohydrases, and lipases. Since excess carbohydrate is stored in the plant largely as starch, even actively growing green plants must be able to digest this compound when it is mobilized for use. There is an even clearer case for digestion in the seeds. Until they reach the light and begin to photosynthesize for themselves, young seedlings are entirely dependent on the foodstuffs stored for their use in the seeds. These include proteins which are needed for synthesis of cell proteins, and varying mixtures of oil and starch. Very little is known about the actual course of plant digestion, but the seeds and seedlings contain the enzymes necessary for digestion of all three types of compounds. In those seeds which store large amounts of oil the lipases are especially abundant; where starch is the chief reserve foodstuff the amylases predominate.

Animal digestion, as was noted in Chapter 3, may be extracellular or intracellular. The latter is not only characteristic of unicellular organisms which customarily engulf their food and then secrete digestive juices into a

food vacuole. This type of digestion is also found in some of the lower multicellular forms, in which the activities of a primitive gut are supplemented by some degree of intracellular digestion. But with the evolution of higher animals the preparation of the foodstuffs for absorption and use became confined to a special set of organs. Obviously the details and the complexity of this digestive system vary from species to species. For example, the compound stomach and the long intestine of the herbivores are especially adapted to their bulky diet and are not found in carnivorous forms. But on the whole the digestive process is the same in all kinds of animals, using the same or very similar enzymes and giving rise to the same split products. For obvious reasons, these reactions have been most extensively investigated in man and other mammals, hence the following description of the organs and enzymes concerned with digestion refers specifically to the human system except where otherwise indicated.

The Digestive System

The relation between the various digestive glands and organs in man was shown in Figure 3.8 (p. 77). With minor variations this arrangement may be considered typical of the vertebrates. While a detailed discussion of the physiology of secretion and enzyme activation is outside the scope of this book, it will perhaps be helpful to outline briefly the various digestive fluids and the enzymes which they furnish.

SALIVA

Saliva is secreted into the mouth from numerous glands but chiefly from the submaxillary, sublingual, and parotid glands. It consists of a dilute solution having a solid content of only about 1.0 per cent, of which a high proportion is the slimy glucoprotein, mucin. This substance contributes to one of the main functions of saliva, that of lubricating both the tissues of the mouth and throat and the food mass, which is thus rendered easier to swallow. The only digestive enzyme in saliva was originally named "ptyalin"; it is an *amylase* which acts upon boiled starch over a wide range of pH values. The pH of saliva itself varies between 6.2 and 7.6.

GASTRIC JUICE

In the lining of the stomach two types of cells contribute to the mixed gastric juice. This is a dilute secretion, containing a mucin-like protein, and also hydrochloric acid, some inorganic salts and several enzymes or pro-enzymes. A conspicuous property of the gastric juice is its acidity. The concentration of hydrochloric acid is approximately 0.12N, corresponding to a pH of about 1.0. After the food enters the stomach it is slowly mixed by peristaltic motion with this strongly acid solution until the pH of the mass is sufficiently low for the gastric proteinases to act.

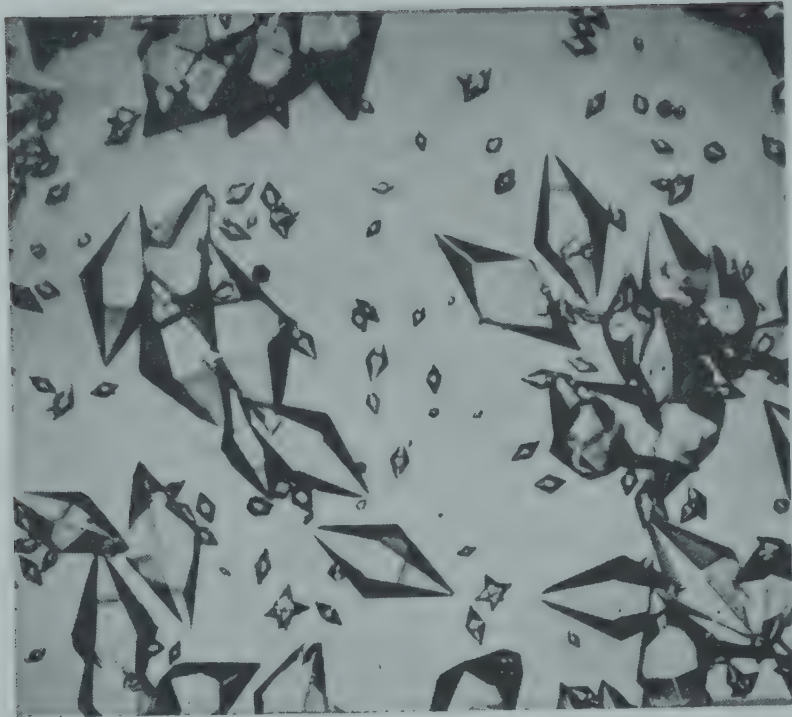
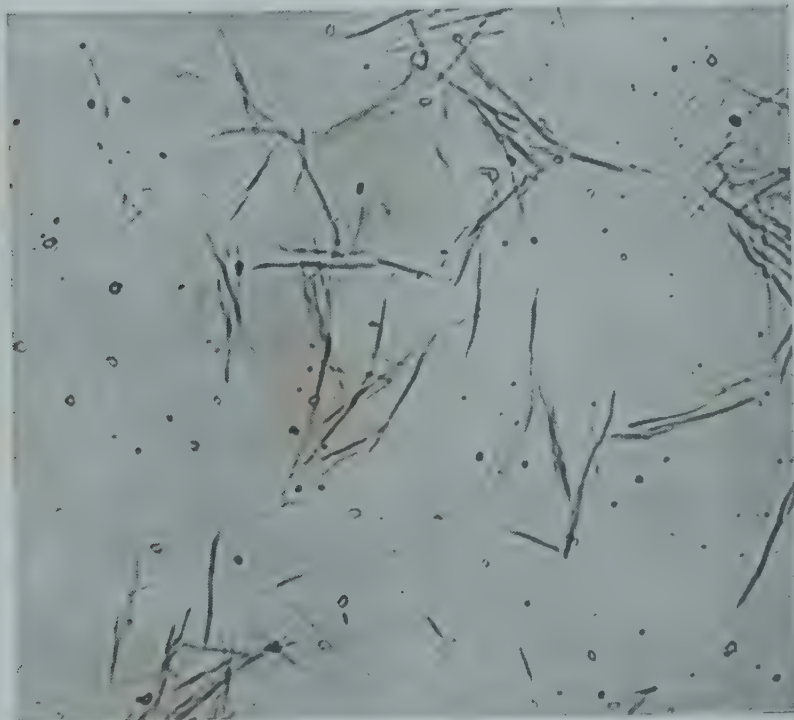


Figure 10.1. Crystals (above and below) of pepsinogen and of pepsin. (From R. M. Herriott, *J. Gen. Physiol.*, 21:501, 1937-38; and R. M. Herriott and J. H. Northrop, *J. Gen. Physiol.*, 18:39, 1934-35.)



The chief enzymes of the gastric juice are the two which act upon proteins. These are *pepsin* and *rennin*, both of which are secreted as inactive zymogens. Pepsinogen becomes the active pepsin when the pH falls to 6 or below. In Figure 10.1 are shown crystals of both the zymogen and the enzyme. The optimum pH for the activated enzyme is close to 2, and thus the high acidity of the gastric juice serves both to activate the zymogen and to furnish a medium in which the activated enzyme can attack the intact proteins which serve it as substrates. In like manner prorennin is activated by a pH of 5 or less. Rennin brings about the coagulation of milk, apparently by causing a partial hydrolysis of its principal protein, casein. One of the products of this hydrolysis is the soluble "paracasein," which forms an insoluble calcium salt. This precipitate makes up the "curd."

There is some evidence that the gastric juice also contains a lipase, but the acidity of the medium is not favorable for lipolytic action. Certainly no extensive hydrolysis of fats takes place in the stomach.

When the food has been thoroughly mixed with the gastric juice, it forms a thick liquid mass, partly solution, partly emulsion, partly suspension, known as *chyme*. After a time, which varies with the individual and with the nature of the meal, a small portion of the chyme is ejected through the pyloric valve into the upper small intestine or *duodenum*. This process is repeated at more and more frequent intervals until the stomach is empty.

INTESTINAL DIGESTIVE JUICES

The acid chyme at first lies in the curve of the duodenum while digestive juices pour upon it; somewhat later constrictions of the intestinal wall begin to mix food and secretions. These secretions arise in three different places, and consist of *intestinal juice*, secreted by glands in the lining of the intestine; *pancreatic juice*, the external secretion of the pancreas; and *bile*, which is secreted by the liver and stored in the gallbladder until needed. All three of these fluids are alkaline in reaction and ultimately bring about neutralization of the acid chyme.

Intestinal digestive enzymes are secreted throughout the length of the small intestine, but by far the largest volume of the intestinal juice is formed in the duodenum. It contains numerous hydrolytic enzymes, including *maltase*, *sucrase*, *lactase*, and *amylase*, several enzymes concerned with the hydrolysis of peptones and proteoses, and others which together bring about the digestion of nucleic acids. *Enterokinase* is the name given to the component of intestinal juice responsible for activation of the pro-enzyme, trypsinogen, which is secreted as the inactive pro-enzyme in pancreatic juice.

Opening into the duodenum just below the pyloric valve is a duct formed by the fusing of two separate ducts from gallbladder and pancreas

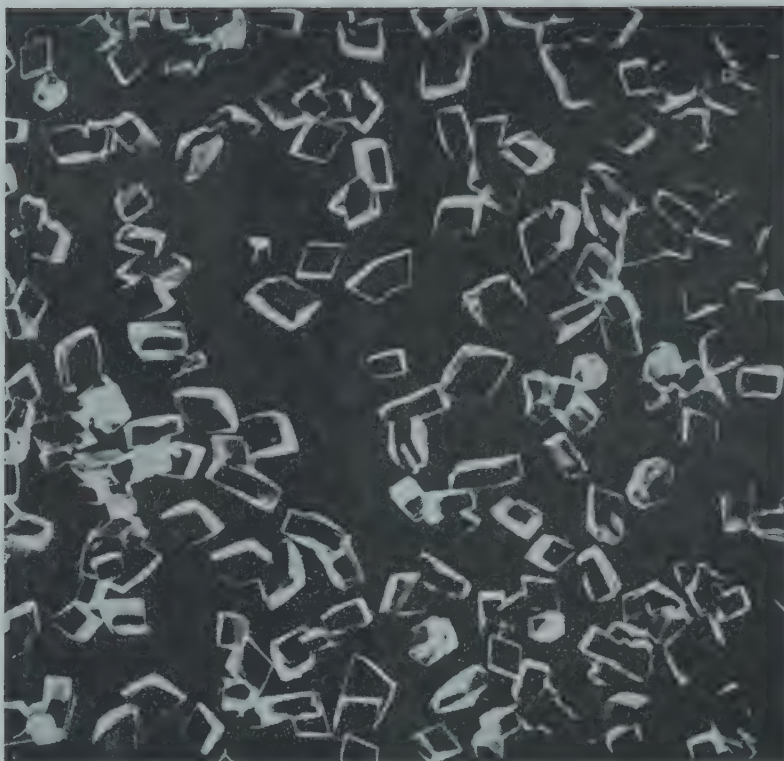


Figure 10.2. Crystals of chymotrypsin. (From M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, 18:446, 1934-35.)

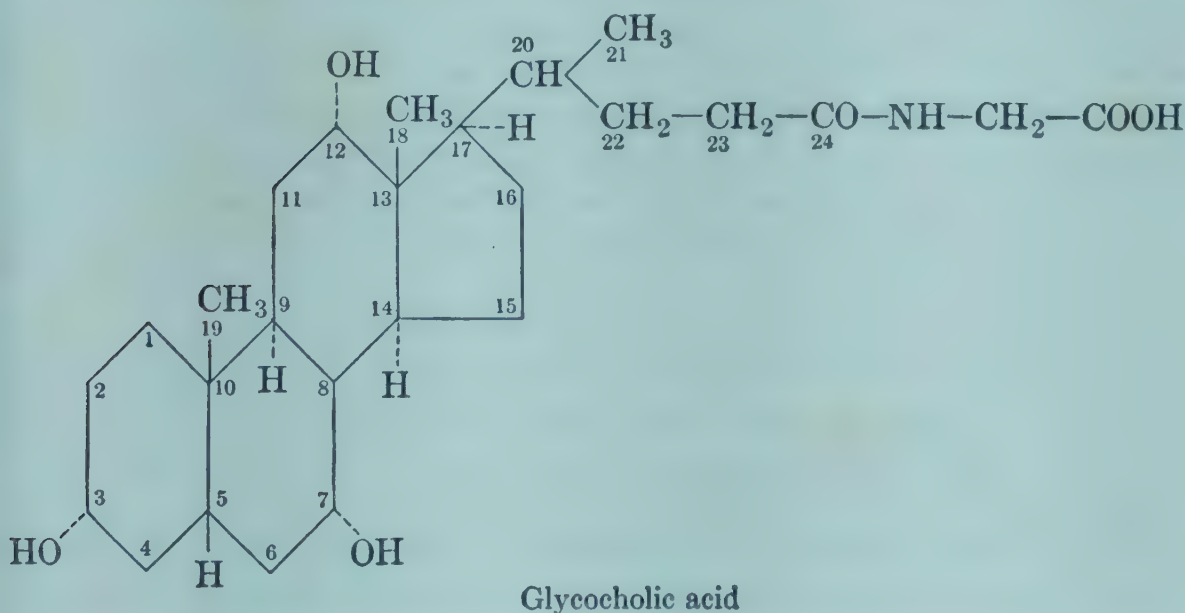
Figure 10.3. Crystalline trypsin. (From M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, 19:994, 1935-36.)

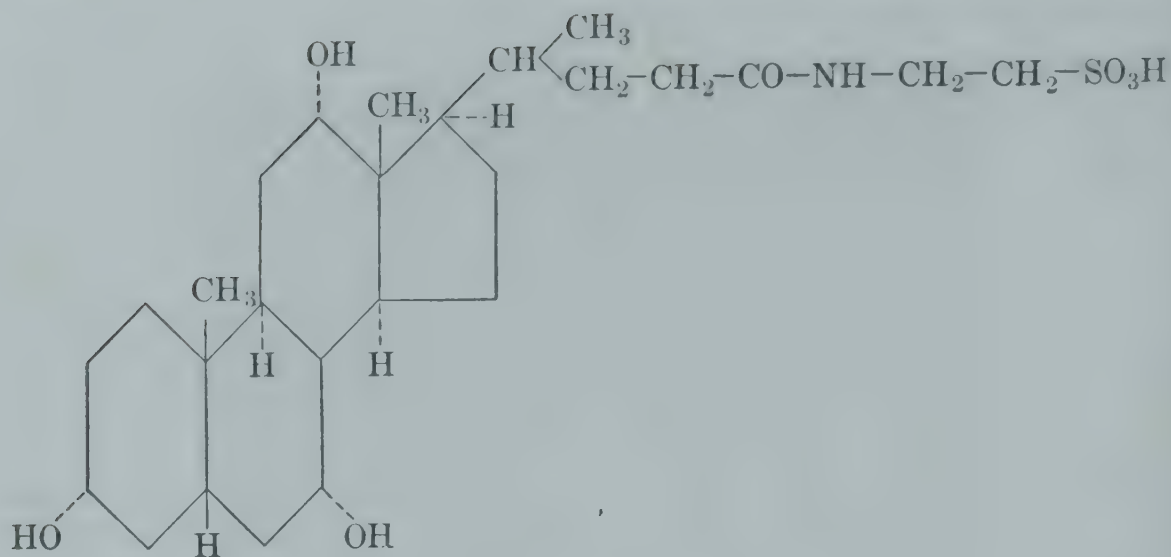


(see Fig. 3.8). Through this passage there is poured over the food mass lying in the duodenum a mixture of bile and pancreatic juice. The latter contains an amylase, known in years gone by as "amylpsin"; a lipase, originally named "steapsin," and at least three different enzymes concerned with protein digestion. Of these last, one is secreted as an active enzyme and is concerned with digestion of polypeptides; the other two are secreted in inactive forms. One of these two, trypsinogen, is activated when it comes in contact with enterokinase of the intestinal fluid, becoming the enzyme trypsin. Chymotrypsinogen, which is activated by the newly formed trypsin, is known thereafter as chymotrypsin. Both enzymes act upon the intact proteins of the foodstuffs and have similar pH optima at about 8. These enzymes and their zymogens are among those which have been crystallized by Northrop and his co-workers, and Figures 10.2 and 10.3 show the crystals of the two active enzymes.

Bile has already been discussed in connection with the destruction of hemoglobin (see p. 303). Mammalian bile contains in addition to the pigments, sodium salts of the two bile acids, *taurocholic acid* and *glycocholic acid*. These are known as the bile salts, and have the property of lowering markedly the surface tension between water and oils. This facilitates the formation from fats of a fine emulsion, which in turn provides a very large surface on which the lipases can act. Furthermore, the presence of these salts causes fatty acids and various otherwise insoluble steroids to go into solution, thus furthering their absorption from the intestine.

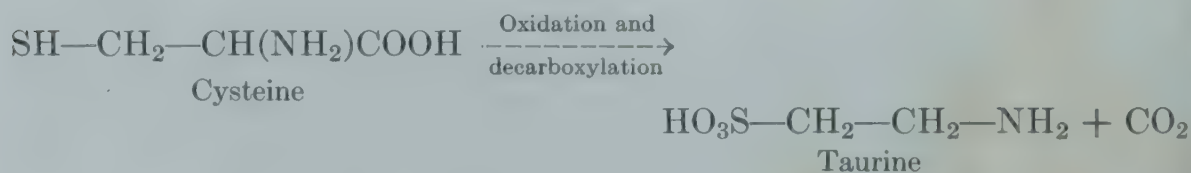
The bile acids are conjugated compounds which are hydrolyzed by heating in acid solution. Glycocholic acid yields glycine and a compound related to the sterols which is known as *cholic acid*; taurocholic acid gives rise to the same cholic acid and to the amino sulfonic acid, taurine, $SO_3H-CH_2-CH_2-NH_2$. The formulas of the bile acids show the close relationship of cholic acid to cholesterol.





Taurocholic acid

Cholic acid is synthesized in the liver, perhaps from cholesterol, though this has not been proved. Glycine is usually available from protein digestion, and in any case is one of the amino acids which can be synthesized by living cells. Taurine presumably arises from cysteine. The experimental evidence for this was obtained in an experiment in which methionine containing radioactive sulfur was administered to animals. Subsequent analysis of the tissues discovered radioactivity in taurine as well as in the more closely related cystine and cysteine.



Although cholic acid is the main steroid component of mammalian bile acids, other closely related acids are known to occur conjugated with taurine or glycine. These acids, like cholic acid, are all hydroxy derivatives of the 24-carbon cholanic acid and differ from cholic acid in the number or positions of the hydroxyl groups. Several of these compounds have been found in small amounts in human bile while others seem to be confined to small groups of related species. A few of the more common acids are listed in Table 10-I.

After being thoroughly mixed with the bile and with the digestive juices of the intestinal tract, the food mass moves slowly along the small intestine. In its passage it meets more enzymes secreted by the mucosa and these join those already present in splitting the foodstuffs into small soluble molecules. In the ruminants there is an interesting provision for making cellulose available as a foodstuff, though the animals themselves elaborate no cellulose-splitting enzymes. Some of the hordes of bacteria

TABLE 10-I. STEROID COMPONENTS OF BILE ACIDS IN VARIOUS SPECIES

Acid	Formula	Position of Hydroxyl Groups	Occurrence
Cholic acid	$C_{24}H_{40}O_5$	3, 7, 12	Man, kangaroo, monkey, mouse, whale, dog, fox, cat, lion, otter, walrus, horse, etc. Many fish and reptiles
Desoxycholic acid	$C_{24}H_{40}O_4$	3, 12	Many vertebrates
Hyodesoxycholic acid (Gr. <i>hyo</i> -, swine)	$C_{24}H_{40}O_4$	3, 6	Hog
Chenodesoxycholic acid (Gr. <i>cheno</i> -, goose)	$C_{24}H_{40}O_4$	3, 7	Many vertebrates including fish
Lithocholic acid	$C_{24}H_{40}O_3$	3	Man, ox, rabbit
Pythocholic acid	$C_{24}H_{40}O_5$	3, 12 and 15 or 16	Python and other members of its species

which inhabit the long gut degrade the cellulose to compounds which can be utilized by the host, thus affording an excellent example of the beneficent coöperation of two organisms which is known as *symbiosis*.

In the large intestine there are few if any new enzymes. Some mucus is secreted, but much water is absorbed, so that the contents of the colon become progressively less watery. Although the feces do finally contain a certain amount of undigested food this makes up far less of the total mass than is commonly supposed. They consist largely of residues from the digestive fluids, together with excretory sterols and pigments secreted in the bile, to which is added a staggering number of bacteria, living and dead. The latter may provide as much as one third of the total dry weight of the feces!

After this brief examination of the topography of the digestive tract, the rest of this chapter will be devoted to a summary of the course of digestion and absorption of the different classes of foodstuffs. It is most convenient to treat each group separately, and within the group to discuss separately the action of individual enzymes. But it must be emphasized that digestion of all the foods goes forward at once except when one enzyme can act only upon the product of another's activity.

Figure 10.4 shows diagrammatically the relation of the blood circulatory paths to the organs of the body and indicates also the course of the main lymphatic ducts. It will be remembered that the lymph system begins in the tissues as blind tubes which fuse to form larger and larger vessels and finally empty their contents into the blood stream. The lymph capillaries in the intestinal mucosa are known as *lacteals*, one of which is found in each of the thousands of tiny villi which jut into the lumen of the gut. Figure 10.5 shows the relation of the blood capillaries of a villus to the

central lacteal with its closed end. When the food molecules are ready for absorption they diffuse into the villi, entering either the blood capillaries or the lacteals. Those which enter the blood are carried directly

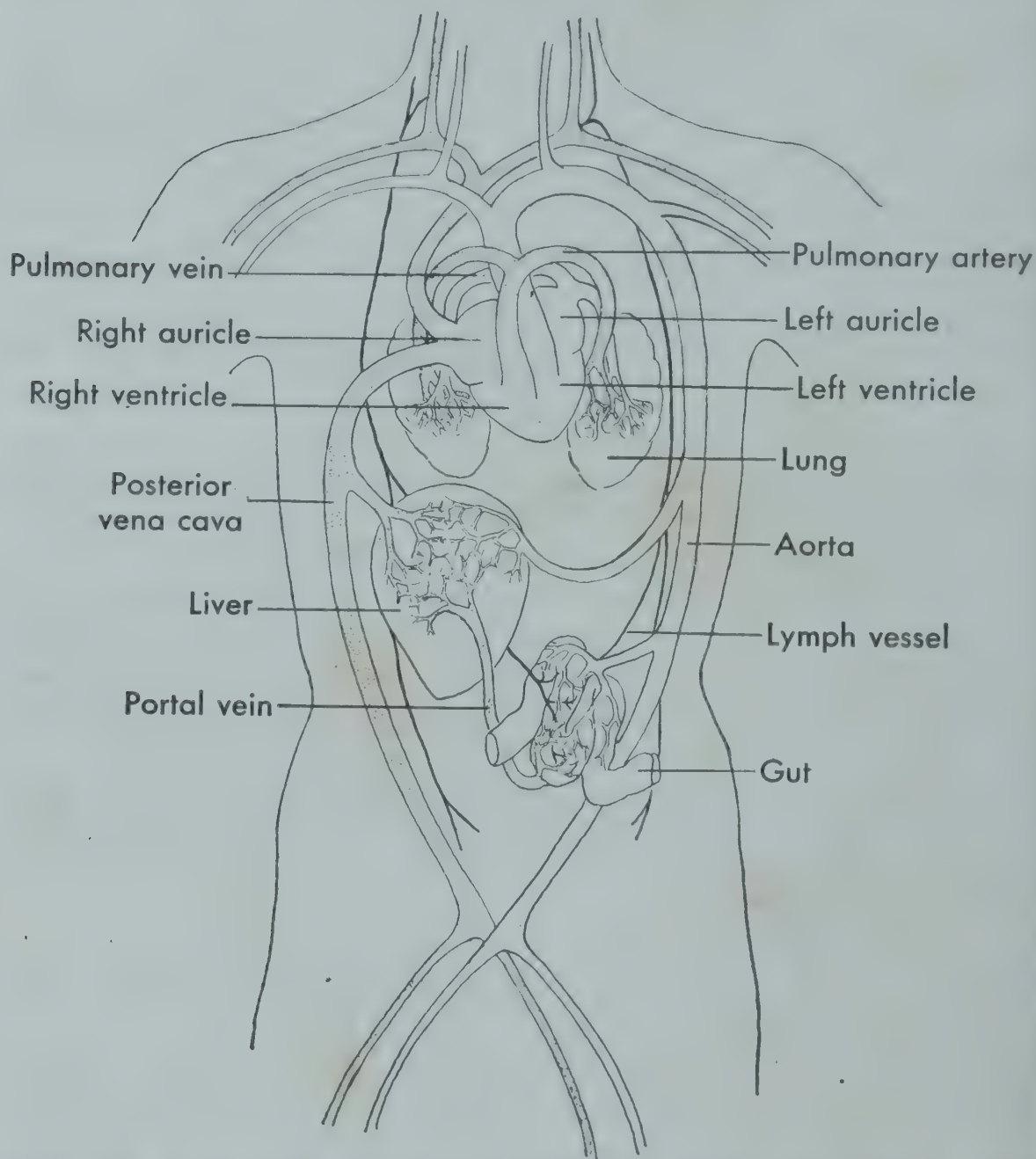


Figure 10.4. Diagram of the blood circulatory system and of the main lymph vessels. The latter are represented by the solid black lines which originate in the tissues and empty finally into the right and left subclavian veins.

to the liver by way of the portal vein. Those which enter the lacteals move much more slowly through the lymphatics and are finally transferred to the general or systemic circulation by way of the main thoracic duct. Thus the liver has first call upon any molecules which enter the capillaries, but those which enter the lacteals reach the liver only indirectly and slowly.

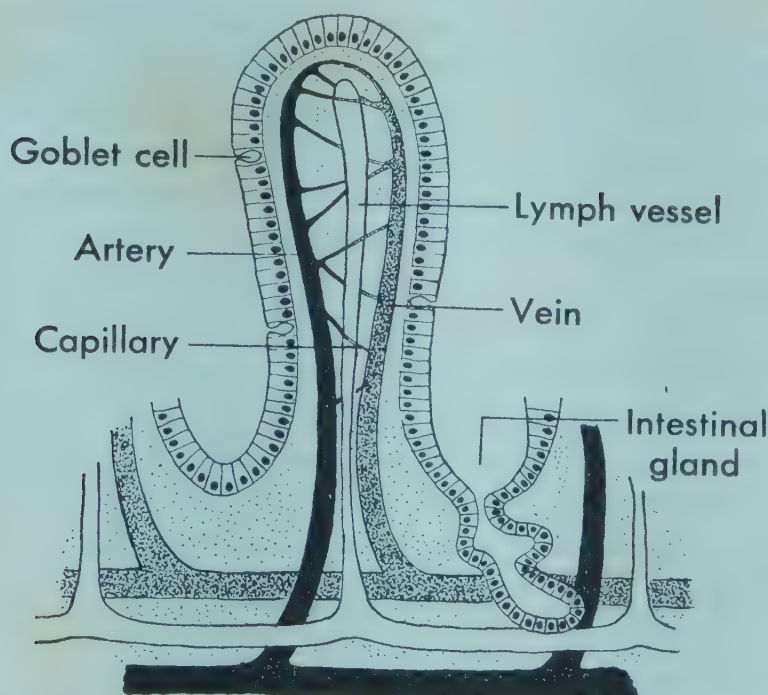


Figure 10.5. Diagram of a villus, showing the blood capillaries and the single lacteal or lymph vessel.

Digestion and Absorption of Proteins

THE PROTEIN-SPLITTING ENZYMES

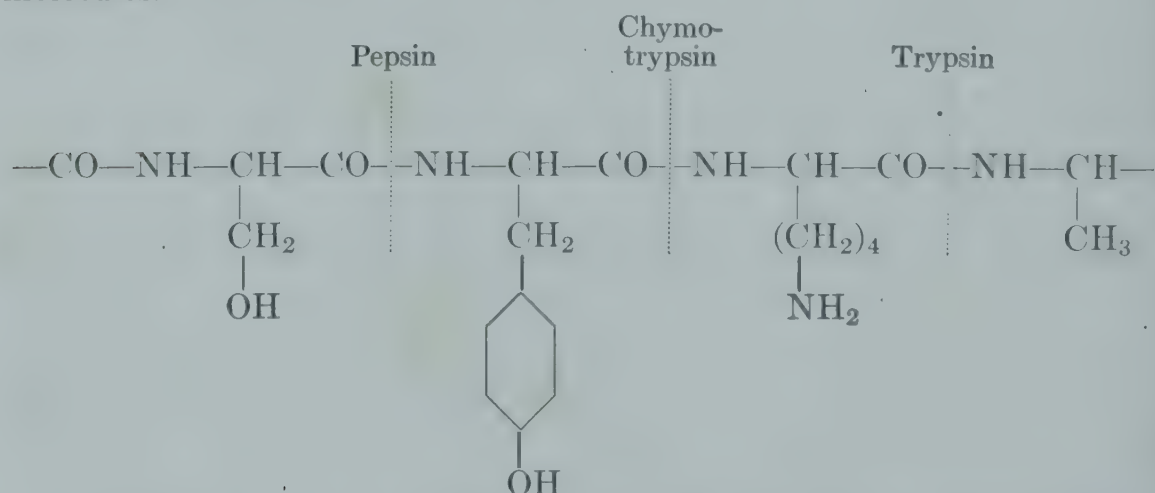
Until quite recently it was believed that two distinct types of enzymes collaborate to bring about the complete hydrolysis of proteins. Pepsin, trypsin, and chymotrypsin represent the group once thought to have the unique ability to attack native protein molecules and to degrade them to proteoses and peptones. The further hydrolysis of these derived proteins, or of the polypeptides derived from them, was supposed to be the function of a single enzyme, called erepsin, found in the intestinal secretion. When later it became evident that several enzymes were involved in the later stages of protein digestion, these were referred to collectively as "erepsins."

A radical revision of this old classification was made necessary by the work of Bergmann and his collaborators. Reference has already been made to the Bergmann method for protecting amino groups, which made possible for the first time the synthesis of a wide variety of peptides (see p. 162). With these synthetic substrates available, Bergmann addressed himself to the problem of the specific requirements of the various protein-splitting enzymes. From his work has come not only an understanding of the specificities of the individual enzymes (see p. 245) but also a completely new classification of the enzymes themselves.

Exopeptidases. It is now clear that certain of the digestive proteases attack only those peptide bonds which hold a terminal acid. If such an enzyme splits an amino acid from that end of the polypeptide chain which

has a free carboxyl group, it is known as a *carboxypeptidase*; if it frees from the other end of the chain an acid whose carboxyl group is involved in linkage, but whose amino group is free, it is called an *aminopeptidase*. These enzymes are known collectively as *exopeptidases*, since they attack only external bonds, and they include all those agents originally designated as erepsins. At one time it was believed that when a polypeptide chain had been degraded to the dipeptide stage a special enzyme concerned itself with the final hydrolysis. This is now somewhat uncertain. It seems more likely from what is known of the specificity of proteolytic enzymes in general that there exist a great number of exopeptidases, each adapted to hydrolysis of bonds between more or less rigidly specified amino acids. Requirements which have to do with that part of the amino acid which is not involved directly in peptide bonds, Bergmann calls "side group specificity." If all the demands of a particular enzyme are fulfilled, it can probably free a particular terminal acid, whether it is attached to a polypeptide or a dipeptide.

Endopeptidases. Pepsin, trypsin, and chymotrypsin differ from the exopeptidases in that they are capable of attacking peptide bonds which are centrally located in the protein molecule. Bergmann classifies these enzymes as *endopeptidases*. As indicated previously (see p. 245) their positional requirements are extremely specific, including both backbone and side chain specificities. Pepsin cannot act if either of the two acids involved in the peptide bond has a free amino group. Trypsin requires such a free group. Pepsin and chymotrypsin attack only bonds which bind an aromatic acid in the chain. For pepsin to act, the aromatic acid must be the one which furnishes the —NH— of the peptide bond; for chymotrypsin the aromatic acid must supply the peptide carboxyl. In the formula below are indicated the points at which pepsin, trypsin, and chymotrypsin might attack a protein, setting free the central tyrosine and lysine molecules.



For trypsin to act, the bond must be formed through the carboxyl group of one of the basic acids, arginine or lysine. Bergmann's work has shown

that the original classification, which limited these enzymes to action upon the intact protein molecule, has no basis in fact. Each will attack any bond which meets its specifications, whether it occurs in a protein or in a much smaller molecule. The property which distinguishes this group is the ability to attack bonds other than terminal ones.

DIGESTION OF PROTEINS

In the stomach pepsin begins the hydrolysis of proteins as soon as the required acidity is reached. This process gives rise to polypeptides of different molecular weights, depending upon where the aromatic acids appear in the chain. Peptic digestion continues as long as food remains in the stomach, and probably goes on locally for varying lengths of time in the upper intestine. The neutralization of the acid chyme is a slow process, and even after peristalsis has begun there are undoubtedly many places in the food mass in which the acidity is sufficiently high for pepsin to act.

Wherever the acidity of the chyme is neutralized, or nearly so, by the alkaline fluids in the upper intestine, trypsin and chymotrypsin take up the hydrolysis of internal bonds. Simultaneously the various exopeptidases of the pancreatic and intestinal fluids are at work, splitting terminal amino acids from the peptide chains. Ultimately all these overlapping hydrolyses result in the complete degradation of the food proteins to a mixture of amino acids. See Table 10-II for a summary of the digestive enzymes which act on proteins.

TABLE 10-II. ENZYMES CONCERNED IN PROTEIN DIGESTION

Substance Secreted	Source	Activating Agent	Active Enzyme	Substrate	Activity Range (pH)	Product
Pepsinogen	Gastric juice	Hydrogen ion	Pepsin	Internal peptide bonds	Varies with substrate 1.5-2.5	Polypeptides
Pro-rennin	Gastric juice	Hydrogen ion	Rennin	Casein	4.0-6.0	Caseinogen
Trypsinogen	Pancreatic juice	Enterokinase	Trypsin	Internal peptide bonds	7.8	Polypeptides
Chymotrypsinogen	Pancreatic juice	Trypsin	Chymotrypsin	Internal peptide bonds	7.6	Polypeptides
	Intestinal juice		Amino peptidases	Terminal peptide bonds		Terminal amino acids
	Pancreatic juice		Carboxypeptidases	Terminal peptide bonds		Terminal amino acids

ABSORPTION OF PROTEINS

Absorption, except for a very few substances, begins in the duodenum and is largely confined to the small intestine. When the food mass has been acted upon by the digestive juices of pancreas and intestine, the proteins have been reduced to a mixture of peptones and smaller peptides

with some free amino acids. Since all of these are water-soluble, there is no obvious reason why they should not all diffuse through the capillary walls and thus enter the portal circulation. But, though there have been some apparent exceptions, the weight of evidence points to the conclusion that proteins are completely hydrolyzed to amino acids before being absorbed. In the early stages of digestion the concentration of peptones and peptides in the intestine is high, but there is no satisfactory evidence that such products pass into the blood stream. During this same time the concentration of amino acids in the blood rises steadily for two and a half to three hours, then gradually falls to the preabsorptive level. This rise in amino acid concentration in the blood runs parallel with a corresponding loss of nitrogen from the gut. As early as 1913 Abel¹ attempted to obtain direct evidence on this point by an experimental method which he named *vividiffusion*. The arterial blood of a living anaesthetized animal was deflected through a series of collodion tubes immersed in saline, before being returned to the animal's own system by way of a vein. Among the substances which diffused into the surrounding saline solution several amino acids were identified, but no more complex nitrogenous molecules were found. A further strong argument against the absorption of peptones is the fact that direct injection of such a mixture into the blood stream gives rise to "peptone shock," a reaction which is absent during normal absorption of a protein meal.

Very recently, making use of new micro methods for the quantitative estimation of amino acids, this whole question has been re-examined. Dogs were fed various kinds of proteins and the amino acid content of both portal and systemic blood was followed. The amino acid concentration was found to increase in the portal blood even more rapidly than in the general circulation, as would be expected if the acids were pouring into the capillaries of the villi. There were found also very slight increases in the concentration of "bound amino nitrogen" in the blood. That is, the number of free amino groups in the serum increased slightly after hydrolysis, as would happen if there were present a small concentration of peptides. However, it was found that this same rise in bound amino nitrogen also followed ingestion of glutamic acid alone. This points to the possibility that the compounds involved arise, not from absorption at all, but from tissue breakdown. In any case, "the results indicate that at most a minor part of the protein was absorbed in peptide form." In this connection it should be noted that this process of absorption is not entirely

¹ Dr. John Jacob Abel (1857-1938) was one of the brilliant group of pioneers who contributed so significantly to the early years of the Johns Hopkins Medical College. It was in his laboratory that adrenaline was first isolated and insulin first crystallized. When he retired in 1932 as Professor of Pharmacology a special department was created in which he continued his scientific investigations until very shortly before his death.

a passive one. This is shown by the fact that the L-amino acids, that is the natural isomers, are absorbed more rapidly than their D-isomers, indicating the existence of some active mechanism which distinguishes between the two forms.

As indicated in the diagram on page 328, the absorbed amino acids are carried directly to the liver via the portal vein. Their fate in this organ and elsewhere in the body is considered in the chapter on Protein Metabolism.

Digestion and Absorption of Fats

Although a lipase is present in gastric juice, the acidity of the stomach makes it very unlikely that there is any gastric digestion of fats in the adult. However, during the time that the food mass remains in the stomach the churning motions by which it is mixed with the gastric secretions probably begin the emulsification of fats. Then in the intestine emulsification is greatly facilitated by the presence of the bile salts. Here also the acidity of the chyme is slowly neutralized so that the lipases of the pancreatic and intestinal juices can act. Under these conditions it is presumed that part at least of the food fats are hydrolyzed, though recent experiments indicate that this is normally so slow a process that hydrolysis does not go to completion. One fatty acid seems to be split off at a time from the triglycerides so that the major products are not free glycerol and fatty acids, but fatty acids and a mixture of monoglycerides and diglycerides.

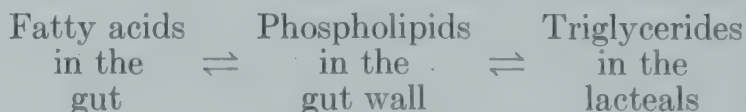
FAT ABSORPTION

The absorption of fats differs from that of the carbohydrates and the proteins in two ways. In the first place both polysaccharides and proteins are hydrolyzed completely by the digestive enzymes, and the resulting monoses and amino acids appear as such in the portal blood. But in spite of the presence of lipases in the digestive tract, the substances which are found in the blood during absorption of a fatty meal are not the split products of fat hydrolysis, but tiny globules of simple fats which are known as *chylomicrons*. It is the presence of this highly emulsified fat which gives to serum from blood drawn an hour or two after a fatty meal its characteristic milky appearance. The other respect in which the fats are unique is in their path of absorption. The carbohydrates and the amino acids diffuse into the blood capillaries and are carried by the portal vein directly to the liver which is thus in a commanding position to regulate their concentration in the general circulation. The major part of the fats on the other hand is absorbed into the lacteals, passing then by way of the lymphatics into the left subclavian vein. They thus enter the systemic circulation directly without being first subjected to any regulatory effect of the liver. There is no obvious reason for the fats' being absorbed by this special path unless it allows the liver, which has to cope with rather com-

plicated problems of fat metabolism, to deal with them more slowly instead of being flooded with them at the peak of absorption.

Mechanism of Absorption. There have been two major theories advanced to account for the presence in lymph and serum of unhydrolyzed fat droplets. There is, as we shall see, a good deal of experimental evidence for each one, but not enough to make either one completely acceptable.

The earlier, or Lipolytic Hypothesis, was advanced and sponsored by Verzár of the University of Basle, who set it forth in some detail in his book *Intestinal Absorption*, published in 1936. He proceeded from the reasonable assumption that the lipases have a function and that the fats are hydrolyzed in the intestine. Since they appear in the lymph, just on the other side of the intestinal mucosa, as complete triglycerides, the theory seeks to explain their re-synthesis. There is some experimental evidence that the phospholipids are involved in this process. For example, the phospholipid content of the intestinal lymph increases during absorption of a fatty meal. It has also been found that when inorganic phosphate labeled with radioactive phosphorus is fed, the radioactivity appears in the phospholipids of the intestinal wall. This indicates a fairly rapid synthesis of these compounds in the cells of the mucosa. A third bit of evidence is the fact that ingestion of fatty acids labeled in one way or another is followed by their incorporation in the phospholipids of the intestinal mucosa and fluids. According to Verzár's theory these facts and other similar ones can best be explained by assuming that the products of fat digestion move into the cells of the intestinal lining and are there built into phospholipids in which form they move toward the lacteals. Somewhere in this short transit a third fatty acid must be substituted for the phosphocholine group, since the major part of the lymphatic fat is simple triglyceride and not phospholipid. It is sometimes suggested that the cells of the gut wall may have a relatively constant supply of phosphocholine which acts as a ferry, carrying successive pairs of fatty acids across the cells of the mucosa. Thus there is postulated an equilibrium between the various lipid constituents in the intestine, in its wall and in the lacteals, an equilibrium which is pushed in the direction of absorption by a concentration gradient when fatty acids are being freed by the lipases.



The second and later theory, known as the Partition Hypothesis, has been sponsored by A. C. Frazer and his colleagues at the University of Birmingham. The experimental basis for their theory may be summarized as follows:

1. Even so unphysiological a substance as liquid paraffin is absorbed into the lymphatics if it is provided in a fine enough emulsion.

2. When olive oil alone is fed to rats there results a general systemic *lipemia*, or increase in the fat content of the blood. This would be expected if the fats reach the circulation by way of the lymphatics. But if olive oil is given mixed with lipase the lipemia is in the portal vein and fat is deposited in the liver instead of in the usual fat depots.
3. When olive oil is acted upon for several hours by lipase, it is not split completely to acids and glycerol but rather loses one fatty acid at a time, leaving monoglycerides and diglycerides.
4. A mixture of a fatty acid and a monoglyceride with bile salts constitutes a particularly effective emulsifying agent for fats.

According to the Partition Theory part of the fats is absorbed without hydrolysis into the lacteals while the products of fat digestion are absorbed into the portal circulation. In these terms the chief importance of lipid digestion is that it provides some free fatty acids and lower glycerides which admixed with bile salts serve to transform the remaining fat into an emulsion having microscopic droplets of such dimensions that they can pass into the lacteals.

There is at present no clear evidence on which to decide whether either or neither or both of these theories is really applicable to the absorption of fat. Recent work with isotopically labeled palmitic acid throws some doubt on the portal absorption of free fatty acids. It was found that whether it was fed in the form of free acid or combined with glycerine as tripalmitin, approximately 70 to 90 per cent of the labeled palmitic acid was recovered in the lymph fluid which was tapped directly with a cannula into the lymph duct. If this result is substantiated it is telling evidence against any significant portal absorption of free fatty acid. All that can be said at present is that although the mechanism of fat absorption still poses unanswered questions there is general agreement that the major part of these substances are normally absorbed into the lymphatic system where they appear as complete triglyceride molecules.

Role of the Bile Salts. The bile salts in the course of a short journey through part of the gut have several functions. Their emulsifying action certainly facilitates the digestion of fats and may well play a part in the absorption of some undigested ones. They also exert what is known as a *hydrotropic* effect on free fatty acids and sterols, causing these substances, normally insoluble in water, to dissolve freely in an aqueous solution of bile salts. This effect depends upon the formation of soluble molecular complexes made up of fatty acid and bile salt in varying ratios. In this form the acids are able to cross cell membranes readily. The bile salts which are not removed from the intestine in this way are ultimately reabsorbed into the circulation and carried back to the liver. Here they are again secreted in the bile and thus launched upon another cycle of activity.

Digestion and Absorption of Carbohydrates

Compared with fats and proteins, the fate of the carbohydrates in digestion is a simple, straightforward one. They are subjected to a series of hydrolytic enzymes beginning with the amylase of the saliva which acts upon boiled but not upon raw starch. The common food polysaccharides, amylose and amylopectin, are degraded by a splitting out of successive maltose units beginning at the outer ends of the various branches. This goes on until every alternate 1-4' link of the branches has been split back to the branching point where presumably a special enzyme intervenes to open the 1-6' or similar link which holds the branch to the main chain. Thereafter maltose units can again be split off from the new open end of the chain. Maltase converts the maltose to glucose, as similar enzymes, invertase and lactase, act upon sucrose and lactose to hydrolyze them to their constituent hexoses. The three monosaccharides glucose, fructose, and galactose then constitute the major part of the absorbed carbohydrate, to which must be added varying small amounts of pentoses from digestion of nucleic acids. There is no evidence that the disaccharides, soluble though they are, can be absorbed without hydrolysis. In fact if a disaccharide is injected into a vein it is promptly excreted in the urine, showing that it is of no use to the cells in that form.

Beyond the fact that disaccharides do not diffuse into the blood, there is further excellent evidence that the passage of carbohydrates into the portal circulation is not simply a passive process, governed by the laws of diffusion. For example, it has been found that hexoses in general are absorbed more rapidly than pentoses, though the smaller size of the pentose molecule would lead to just the opposite expectation. Furthermore the different hexoses are themselves absorbed at widely different rates. In one experiment Cori showed that if the rate at which 50 per cent glucose disappears from the gut is taken as 100, the absorption rates for some other hexoses are as follows: D-galactose 110, D-fructose 43, D-mannose 19. But if these same sugars are injected into the peritoneum, from which they diffuse into the blood without passing through the intestinal mucosa, they all enter the blood at the same rate. This is true also if they are enclosed in a loop of dead intestine and allowed to diffuse *in vitro*. Clearly then the normal diffusion into the villi is an active process governed in some way by the living cells of the intestinal mucosa.

We shall discover when we consider the metabolic changes which the carbohydrates undergo that phosphate derivatives are important intermediates at several different points in the sequence. Since the synthesis of these compounds involves the uptake of energy it can proceed only when coupled with some reaction which yields energy. It has therefore been suggested that the selective absorption of the sugars depends upon the ability of living cells to phosphorylate them at the expense of some

oxidative procedure. This would explain the fact that dead intestine exercises no selective power. This view that carbohydrate absorption must be accompanied by phosphorylation was originally propounded by Verzár on the basis of experiments with enzyme inhibitors, but it has recently received somewhat more direct confirmation. It has been shown that there is a close correlation between the absorption rates of different sugars and the rates at which they are phosphorylated under the influence of the enzymes of finely ground rat intestine. The present situation then is that the phosphorylation hypothesis of selective carbohydrate absorption is the only one in the field and is widely accepted though it is based on indirect evidence and reasoning from analogy.

Summary

As a result of the action of the digestive enzymes and of absorptive processes—some of which are still unexplained—the foodstuffs are transferred to the blood as relatively simple molecules. The proteins appear as α -amino acids and the carbohydrates as hexoses and pentoses. The chylomicrons in the blood stream are chiefly droplets of triglycerides though there are of course small amounts of the compound lipids, of cholesterol both free and esterified with fatty acid, and of the various products of fat digestion. These then are the substances which are at the disposal of the cells and which they remove from the blood as it passes through the capillaries. The chemical reactions which these compounds then undergo, whether of degradation or synthesis, together constitute what is known as their “metabolism.” The remaining chapters of this book will trace, as far as it is known, the story of the complex, interlocking chemical reactions by which the cells obtain energy and maintain their own protoplasmic structures.

Suggestions for Further Reading

GENERAL

DUNCAN, G. G. (ed.), *Diseases of Metabolism*, Saunders, Philadelphia, 1947.

The chapters which deal with the metabolism of the foodstuffs include excellent summaries of their digestion and absorption.

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Study Questions

1. Distinguish between an exopeptidase and an endopeptidase.
2. What point in a polypeptide chain is attacked by a carboxypeptidase? by an aminopeptidase?
3. Give an example of "side group specificity" of a protease; of backbone specificity.
4. What is the experimental evidence for the belief that proteins are absorbed only after complete hydrolysis?
5. What is the experimental basis for the belief that hydrolysis of fat in the intestine is not complete?
6. What is believed to be the main pathway of fat absorption?
7. Sketch briefly the theories of fat absorption proposed by Verzar and by Fraser, indicating the experimental basis for each.

8. Indicate the functions of the bile salts. What is the structure of these compounds?
9. What experimental evidence is there that absorption of carbohydrates is an active and not a passive process as it occurs normally in a living organism?
10. List the compounds which are made available to the cells of an animal by the processes of digestion and absorption.

Metabolism of the Compounds of Nitrogen

Only the great discoverer has at the same time the gift of selecting the significant sign, the power to pursue and the patience to follow to the end the trail which it indicates.

E. N. DA C. ANDRADE (1952)

An adequate supply of nitrogen is essential for plants, for animals, and for microorganisms. Most higher plants obtain their nitrogen in the form of ammonium salts and nitrates of the soil solution. These compounds are taken into the root hairs and the nitrates are reduced by plant cells to the level of ammonia. In some plants this reaction takes place almost entirely in the roots; in others part of the nitrate is translocated to other areas of the plant before being reduced. The individual steps in the formation of ammonia are not known, but the reaction usually proceeds only when accompanied by a parallel oxidation of carbohydrates which thus furnish the energy for the reduction of nitrate groups. The major part of the ammonia which is formed is used by the plant in the synthesis of proteins. As far as we now know many of the processes of amino acid synthesis and degradation follow similar paths in plants, in animals, and in microorganisms.

Since the metabolic reactions of animals have been most extensively investigated the following discussion of nitrogen metabolism centers about those organisms, but it should be kept in mind that many of the enzymatic reactions involved are shared by a wide variety of different types of cells. Plants and many microorganisms are able to use the nitrogen of simple inorganic compounds for protein synthesis, which means for synthesis of enzymes as well as of structural proteins. Animals must be provided with organic compounds of nitrogen which they normally ingest as proteins. But even these organic compounds yield ammonia in the course of metabolism and thus in the upshot all types of cells have very similar material to work with and, as we shall see, very similar enzymic machinery with which to deal with it.

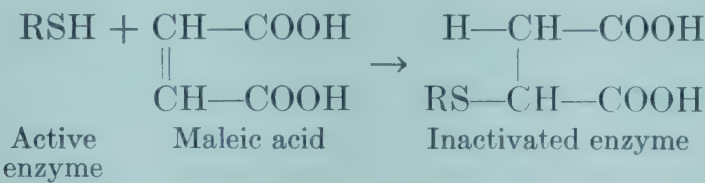
Protein-Splitting Enzymes

We have already seen that enzymes are secreted into animal digestive tracts where they act in sequence to hydrolyze proteins to amino acids.

and that similar enzymes, known as cathepsins, are formed within the cells. Proteolytic enzymes are also elaborated by plants and microorganisms. They have properties very like those of the enzymes of animal origin and presumably serve similar purposes, both hydrolytic and synthetic.

PLANT PROTEOLYTIC ENZYMES

Many plant proteases occur in the latex, which is the milky fluid which forms in special latex vessels and is exuded when the plant tissue is injured. Other similar enzymes are found in seeds and seedlings, where their activity during germination suggests that they may be used to digest reserve proteins and thus to put the soluble amino acids at the disposal of the growing seedling. It is of interest that many of these enzymes share the property which characterizes a number of enzymes of animal origin, of being inactivated by oxidation and reactivated by reduction with sulfurous acid or with sulfhydryl compounds such as cysteine. This is interpreted to mean that all these enzymes depend for their activity upon the presence in the enzyme protein of free sulfhydryl groups. This is indicated both by the parallelism between their activity as enzymes and the amount of free sulfhydryl group as shown by the nitroprusside test, and by their inhibition by maleic acid which forms an addition compound with the sulfhydryl group.



proteins and they exhibit the extreme type of linkage specificity which characterizes the similar enzymes from animal sources.

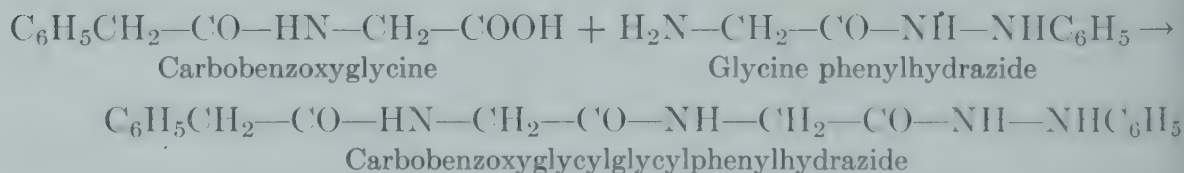
BACTERIAL PROTEOLYTIC ENZYMES

Some but not all groups of bacteria hydrolyze proteins, a property which is often indicated by their ability to liquefy gelatin. This hydrolysis is usually extracellular, that is, the bacteria secrete proteases into the medium and thus are able to disrupt protein molecules to fragments of diffusible size. This process is thus in method, and presumably in purpose, analogous to extracellular hydrolysis of food proteins by digestive enzymes.

Some microorganisms also elaborate intracellular enzymes which act to split peptide bonds. In general these are formed relatively late in the growth cycle and act upon small molecules. How far they exhibit the rigorous specificity of the animal peptidases is not known.

PEPTIDE BOND SYNTHESIS BY ENZYMES

It has been noted before that most enzymic reactions are reversible even though for many enzymes the equilibrium lies far to one side. It is therefore not surprising to find that both plant and animal proteolytic enzymes have proved capable not only of rupturing but of forming peptide bonds. This has been accomplished, for example, by allowing chymotrypsin to act upon a protein-free peptic digest which has been diluted with saline. It is suggested that salt in the concentration used acts to inhibit the digestive function of the enzyme and so allows a "protein-like" condensation product to form and precipitate. In another type of experiment glycylglycine was prepared indirectly through the use of papain as catalyst. Two substitution products of glycine were prepared, the phenylhydrazide in which the carboxyl group is masked, and the carbobenzoxy derivative in which the amino group is protected. These two were then mixed and allowed to stand in the presence of papain. At the end of forty days a precipitate was isolated which proved to be a condensation product of the two derivatives.



Removal of the substituting groups yielded glycylglycine. Thus the ability of proteolytic enzymes to act in a synthetic capacity has been clearly established.

There are however various reasons for doubting that protein synthesis in the living cell is a simple reversal of hydrolysis. In the first place rupture of a peptide bond involves a decrease in free energy of approximately 3500 cal. per mole. The coupling of amino acids can therefore

only take place if a corresponding amount of energy is made available by simultaneous *exergonic* or energy-releasing reactions. The way in which such *endergonic* or energy-using syntheses are normally carried out in the cell has been most extensively investigated in the field of carbohydrate chemistry. There it has been found that the synthesis follows an indirect path and involves not the simple sugars but phosphorylated derivatives. It seems likely therefore that peptide bond formation, which also requires expenditure of energy, may prove to be an indirect synthesis.

Relation Between Food and Body Proteins

In the normal animal diet protein is the source of nitrogen. As this material is metabolized, much of the ingested nitrogen appears in the urine as urea, with smaller quantities of ammonia, creatinine, and uric acid. A further small quantity of nitrogen, eliminated in the feces, is clearly not of dietary origin, since it is present even when the diet contains no protein. Table 11-II gives the composition of a twenty-four-hour specimen of the

TABLE 11-II. RELATION BETWEEN DIETARY NITROGEN AND NITROGEN EXCRETION ^a

Composition of Food (g.)	Composition of 24-Hour Urine											
	Total N		Urea N		Ammonia N		Uric Acid N		Creatinine N		Undeter- mined	
	g.	%	g.	%	g.	%	g.	%	g.	%	g.	%
Protein (118 = 19 g. N)	16.8	100	14.7	87.5	0.49	3.0	0.18	1.1	0.58	3.6	0.85	4.9
Starch (148)												
Carbohydrate (225)												

^a Data from O. Folin, "Analysis of 30 'Normal' Urines," *Am. Jour. Physiol.*, 13:45, 1905.

urine of a man on a normal diet. In this case nearly as much nitrogen has been excreted as was ingested in the twenty-four-hour period, and of this approximately 90 per cent appeared as urea. When the diet is low in protein the amount of nitrogen excreted may be far greater than that ingested, the excess representing nitrogen obtained by catabolism of the body proteins. When the amounts of nitrogen ingested and excreted in a given period are equal, the animal is said to be in nitrogen equilibrium.

ESSENTIAL AMINO ACIDS

Certain proteins, if used as the sole source of nitrogen in the diet, proved many years ago to be inadequate to maintain body weight and health. For example, young rats fed a diet in which zein, the principal protein of corn, was the only source of nitrogen, steadily lost weight. When tryptophan was added the animals maintained their lowered weight, but failed to gain. When zein in the diet was supplemented with both lysine and

tryptophan, the rats began to grow, as shown in Figure 11.1. Similar experiments with other inadequate proteins have established the fact that certain of the amino acids must be furnished in the diet, while others can apparently be synthesized. Rose¹ has defined an *indispensable amino acid*

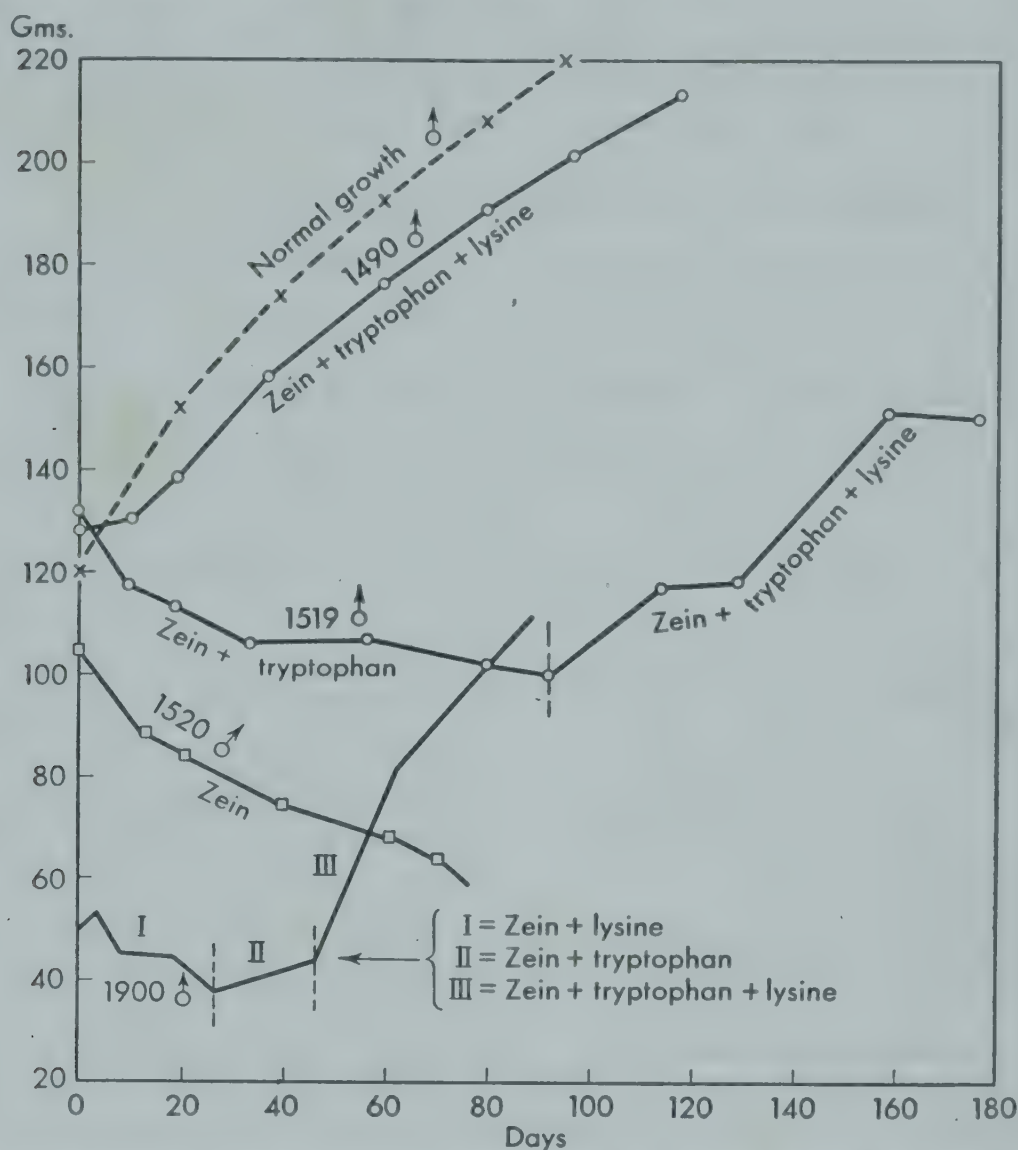


Figure 11.1. Growth curves for rats on diets in which zein was the only protein. On zein as the sole source of nitrogen there was progressive loss of weight. When the diet was supplemented with lysine alone or with tryptophan alone the loss was smaller, and with some animals body weight was maintained. With additions of both lysine and tryptophan the growth curve was very nearly normal. (Data from T. B. Osborne and L. B. Mendel, *J.B.C.*, 17:325, 1914.)

as "one which cannot be synthesized by the animal organism out of the materials ordinarily available at a speed commensurate with the demands for normal growth." In Table 11-III are listed the acids which are indispensable for adult human males. Earlier experiments with small animals had indicated that histidine and arginine were also essential acids.

¹ William S. Rose (1887-) is Professor of Biochemistry at the University of Illinois. He is responsible for most of the recent work in which the amino acid requirements of animals are being defined.

TABLE 11-III. AMINO ACIDS ESSENTIAL FOR MAN ^a

Valine	Methionine
Leucine	Phenylalanine
Isoleucine	Tryptophan
Threonine	Lysine

^a Data from the series of papers by W. C. Rose and his colleagues listed in the Bibliography for this chapter. The requirements for rats are the same except that for them histidine is also a dietary essential.

at least for optimal growth. Rose's experiments have shown that they need not be furnished in the diet of human adults if the other eight acids are present in sufficient quantity.

EARLY METABOLIC THEORIES

Since the time of Lavoisier, various theories have been evolved to explain the food requirements of the living organism. Food was first compared to the fuel used by an engine, the living organism thus constituting the working parts of the machine. Not until the early years of the present century was it shown that the character of the "fuel" is of importance; that proteins must be included in a diet, and that among proteins, only those containing certain specific amino acids are entirely adequate. From these facts Folin ² deduced that the working parts of the bodily machine, consisting in large part of protein, were subject to continual wear and tear, and must therefore require renewal. These working parts were conceived to be essentially stable structures, from which bits were worn out and discarded from time to time. When this happened the organ was believed to replace the lost material from foodstuffs, which must therefore provide a supply of all amino acids that could not readily be synthesized. According to this theory, nitrogen of the food in excess of that required for bodily repair in any given time interval, would be excreted. The metabolism of all those molecules derived from the food was referred to as *exogenous* metabolism.

Those reactions by which the structural elements of the body organs were degraded to yield excretory products constituted the *endogenous* metabolism. In an adult animal, maintaining a constant body weight, the endogenous metabolism was believed to be small. Thus the bulk of the nitrogen in the urine would normally come directly from the food. Obtaining evidence for or against such a theory was, until very recently, beset with insuperable difficulties. Amino acids of the food, once absorbed into the blood stream, were indistinguishable from amino acids broken

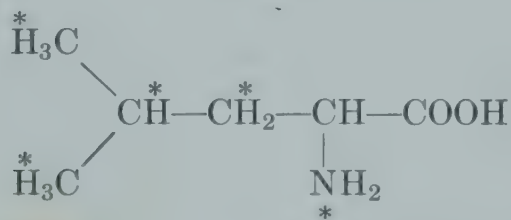
² Otto Folin (1867-1934) was Professor of Biological Chemistry at Harvard from 1909 until his death. He was born in Sweden and came to America at the age of fifteen. He worked in the field of nitrogen metabolism, but is most widely known for the many analytical methods which he and his students perfected.

out of body proteins. Not until isotopic N^{15} became available about 1938 was direct attack upon the problem possible.

THE STATE OF BODY PROTEINS

The work of Schoenheimer³ and his colleagues, using isotopic nitrogen (N^{15}) and deuterium to label the molecules of individual amino acids, has shown that the theory of separate exogenous and endogenous metabolism is completely untenable. Far from consisting of stable structures, repaired only when parts have been "worn out," the living organism is in a continual state of flux. All its proteins are being constantly and rapidly degraded in whole or in part, and as rapidly reconstituted. The free amino acids present in the tissues and in the blood at any given time may have been set free from tissue proteins, or may have come from the foods. Some may even have been in and out of the structural proteins several times.

Schoenheimer obtained the first experimental evidence against the Folin theory when he fed to adult rats L(—)-leucine, labeled with both deuterium and N^{15} , and glycine marked with the nitrogen isotope. The stars in the formula indicate the positions of the isotopic atoms in the leucine.



Leucine

According to the earlier concept, the urine collected during the experimental feeding period should have contained most of the labeled dietary nitrogen. Table 11-IV indicates the actual distribution of N^{15} between excreta and the body proteins. Although there was no increase in the body weights, more than 50 per cent of the isotopic nitrogen administered in leucine had been incorporated into the body proteins, and less than 30 per cent had been excreted. Similar results have been obtained with a number of other labeled amino acids, indicating that the incorporation of dietary amino acids into the body proteins is far more constant and rapid than had previously seemed likely. Analysis of separate organs and of body fluids showed that the isotopic nitrogen had been built into every organ investigated, as well as into serum proteins, hemoglobin, and skin proteins.

³ Rudolf Schoenheimer (1898–1941) was born in Berlin and began his scientific career in Germany. In 1933 political events in Germany forced him to leave his Freiburg laboratory. From that time until his death he worked in the Department of Biochemistry of Columbia University Medical College, and it is from that laboratory that his fundamental work with isotopic tracers was published.

TABLE 11-IV. FATE OF AMINO NITROGEN IN NORMAL ADULT RATS ^a

Isotopic amino acids, corresponding to 25 mg. N per day for 3 days, were added to normal stock diet.

Material Analyzed	Per Cent of Administered N ¹⁵ Recovered	
	After Feeding L(-)-Leucine (%)	After Feeding Glycine (%)
Excreta		
Feces	2.2	2.6
Urine	27.4	40.8
Animal body		
Nonprotein N	8.2	11.1
Protein N	56.5	44.3
Total	94.3	98.8

^a Table from R. Schoenheimer, *The Dynamic State of Body Constituents*, Harvard University, Cambridge, 1949.

Even more surprising were the results obtained when these same body proteins were hydrolyzed, in order to determine the distribution of N¹⁵ among the different amino acids. After feeding isotopic leucine, N¹⁵ was found, not only in the leucine of the organ proteins, but *in every other amino acid estimated except lysine*. The double labeling of leucine made it possible to say positively that its carbon chain as well as its nitrogen had been incorporated into the tissue proteins. Table 11-V gives the N¹⁵

TABLE 11-V. N¹⁵ CONTENT OF AMINO ACIDS ISOLATED FROM PROTEINS OF RATS GIVEN LABELED L-LEUCINE ^a

Recoveries are calculated for 100 atom per cent N¹⁵ in the labeled compound administered.

Amino Acid	Liver	Intestinal Wall	Muscle and Skin
Leucine	7.95	7.35	1.90
Glycine	0.74	0.63	
Tyrosine	0.50	0.94	0.20
Glutamic acid	1.85	2.97	0.89
Aspartic acid	1.16	2.30	0.70
Arginine	0.89	0.43	0.25
Lysine	0.06	0.07	
Amide N	0.78	1.24	

^a From R. Schoenheimer, *The Dynamic State of Body Constituents*, Harvard University, Cambridge, 1949

content of the amino acids isolated from the organ proteins of the rats in this experiment. Of the total N¹⁵ found in the proteins, only 30 per cent was still attached to the leucine residues, and the remaining 70 per cent was distributed among several other amino acids. Of these the

dicarboxylic acids had been most active in acquiring new nitrogen. The presence of the nitrogen isotope in leucine itself is not unexpected, but its presence in significant amounts in so many other amino acids indicates an undreamed of lability on the part both of the amino acids and of the structural proteins. Not only has N^{15} been transferred from leucine to at least five other amino acids, but these acids have all replaced other, presumably similar acid residues previously part of the organ proteins. This process involves the opening of at least two peptide links for each acid residue introduced, and their rapid re-formation to hold the new acid. Similar experimental results with other labeled amino acids lead to the belief that the transfer of nitrogen from one amino acid to another (excepting lysine) goes on continuously, and that there is a constant, rapid interchange between the acid residues of body proteins and the pool of free amino acids. It has been estimated that at least 10 per cent of the liver protein of a rat is newly formed in the course of three days and that one half of it has been resynthesized in about seven days.

Until Schoenheimer published his results it was tacitly assumed that synthesis in the animal body takes place only to fill some specific need for a particular compound. This is far from the truth. In one experiment, tyrosine labeled with N^{15} was added to a diet in which enough casein was provided to meet abundantly all amino-acid requirements. In spite of this abundance of unlabeled acids, isotopic nitrogen was only partly excreted, and that which was retained was found to have been incorporated in all the amino acids isolated, again excepting lysine. As in previous experiments, a high percentage of the isotope had been used in the synthesis of glutamic acid, although there was already available a generous supply of this acid from the casein. Evidently the body constantly builds dietary nitrogen into nearly all the amino acids, regardless of whether there is a lack or not.

Out of these experiments has come a new theory of the nature of adult protein metabolism. The body proteins, including those of the blood and of the tissue enzymes, are apparently extremely labile. Peptide and other bonds are constantly opening and allowing the free amino acids to escape and to merge with those from the foodstuffs. The depleted proteins must simultaneously take up whatever acids they need to replace those just lost, for they themselves remain constant in amount and composition. It is presumably with this continual hydrolysis and resynthesis of peptide bonds that the cellular proteases, known collectively as *cathepsins*, are concerned. During life the synthetic process balances the destructive: after death the hydrolytic reaction is not followed by resynthesis and autolysis of the tissue results. In the living animal the free amino acids, present in both blood and tissues, constitute a constantly changing metabolic pool in which it is impossible to distinguish the source of any particu-

ar acid. From this pool some acids are withdrawn for synthetic purposes. Others are degraded, and their nitrogen excreted. Some exchange their nitrogen for that of other acids, after which they may go back into the protein from which they have come, or be carried elsewhere to enter a totally different protein. Thus Schoenheimer's work has revealed in broad outline the elaborate dynamic equilibrium maintained by living cells. For light upon the specific chemical reactions involved in this system we must turn to other studies and seek answers to such questions as the following: How is nitrogen removed from an amino acid, and where does this removal take place? In what types of synthetic reactions is this nitrogen used? In what form is it finally excreted? How is nitrogen transferred from one acid to another?

Metabolism of the Amino Acids

The first quarter of the twentieth century was marked by a spate of papers concerned primarily with the site of urea formation and to a lesser extent with the whole question of amino acid metabolism. The amino acids of the foodstuffs, absorbed into the capillaries of the villi, pass by way of the portal vein directly to the liver. There many of them are removed from the blood, and at the peak of absorption the amino acid content of the liver may be as high as five times its normal resting value. During the ensuing three or four hours, the amino acid content of the tissue drops, and simultaneously the amount of urea in the blood rises. During this same period there is no such fluctuation in the amino acid content of other tissues. However, all tissues do contain a certain amount of free amino acid, and this raises the question whether the metabolism of amino acids is a function of all tissues, or is restricted to the liver.

ROLE OF THE LIVER

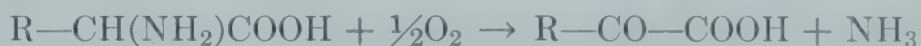
For many years it was impossible to decide where the amino acids are metabolized, because many of the analytical methods and experimental techniques were too insensitive to detect the differences which must be measured. From the great volume of literature on the subject, the following experimental results indicate how the question was finally answered. Perfusion experiments showed that the surviving mammalian liver could form urea from a wide variety of amino acids added to the perfusion fluid. Urea was also formed when the liver was perfused with a solution which included ammonia, and in this case as well as the former one, the synthesis depended upon effective aeration of the perfusion fluid. Thus it was clear that the transformation either involved an oxidative step or required a source of energy. Clinical studies showed that in patients with extensive liver damage, urea excretion decreased and simultaneously the excretion of ammonia increased. Finally in 1924 came the now classical

paper of Bollman, Mann, and Magath,⁴ reporting on the results of complete extirpation of the liver in dogs. This delicate operation was performed in three stages, so that a subsidiary circulation around the liver was developed before the final hepatectomy. The animals recovered from the third operative procedure promptly and were able to survive for four to thirty-four hours if given glucose intravenously. During this time blood and urine analyses were carried out at intervals and the results were compared with those from carefully chosen control animals. It was found that as long as urine secretion was maintained, the urea content of both blood and urine fell progressively, and that there was a simultaneous increase in urinary ammonia. If urinary flow was interrupted, the urea in the blood remained at a constant level and the amino acid concentration rose. These observations, coupled with what was already known, indicated the probable over-all course of amino acid metabolism in the intact mammalian animal. Partly because of its strategic position, the liver removes most of the amino acids which enter the circulation from the gut. The amino groups of some of these acids are rapidly split out as ammonia and from this ammonia urea is synthesized by the liver. The other organ which readily removes ammonia from the amino acids is the kidney, and it is likely that in the intact animal some of the ammonia from which urea is formed by the liver, is in the first place set free from amino acids in the kidney.

DEAMINATION

The removal of the amino group from an amino acid is known as *deamination*. Living cells are able to accomplish this in several different ways, but the reaction which seems to be most fundamental both in plants and animals is the one in which deamination is accompanied by oxidation.

Oxidative Deamination. The history of our increasing understanding of the oxidative removal of amino groups illustrates admirably the use of many of the different biochemical research techniques. As early as 1928 Neubauer⁵ began the study of amino acid metabolism, using acids labeled by phenyl substitution. Feeding of these compounds to animals was followed by excretion of the corresponding aromatic keto acids in the urine. Neubauer suggested then that the first step in amino acid metabolism gives rise to keto acid and ammonia according to the equation:

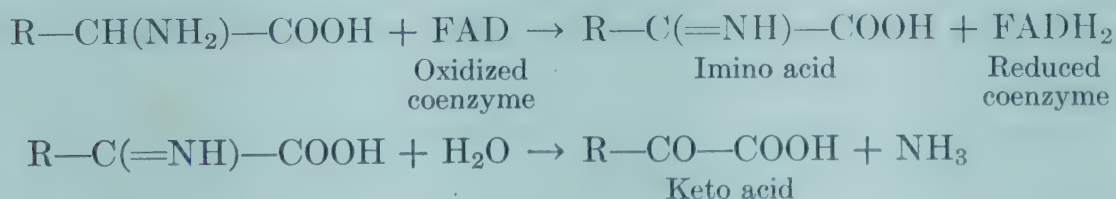


⁴ Dr. Frank Charles Mann (1887–) is Professor of Experimental Medicine at the Mayo Foundation in Rochester, Minnesota, and Dr. Jesse L. Bollman (1896–), Professor of Experimental Pathology. Theirs has been a long and fruitful collaboration concentrated largely in the field of nitrogen metabolism.

⁵ Dr. Otto Neubauer (1874–) was Professor of Internal Medicine at the University of Munich. Since 1942 he has been practicing medicine in England.

A few years later Krebs,⁶ made an exhaustive study of this reaction. He made use of the tissue slice technique and determined both the Q_{O_2} and the Q_{NH_3} , besides isolating the keto acids in some cases. This study showed that mammalian kidney is the most active deaminating organ in the body, setting free ammonia three times as fast, per gram of dry weight, as the next most active organ, the liver. It should be noted, however, that because of its much greater weight the liver actually deals with the larger proportion of amino acids. Krebs was able to prove that the kidney contains two separate deaminating systems, one of which oxidizes and deaminates the natural or L-amino acids, while the other attacks only the unnatural stereoisomers. Under normal conditions the keto acids which are formed by either system are promptly oxidized or otherwise changed. In order that they may accumulate in sufficient quantity to be isolated, it is necessary to poison the enzymes responsible for their oxidation. Krebs was able to achieve this by the use of arsenic trioxide, in the presence of which the deamination proceeds smoothly, while the further oxidation of the resulting keto acids is blocked. Under these conditions the keto acids accumulated in such quantity that it was possible to isolate them as their 2,4-dinitrophenylhydrazones. In some of the experiments the rate of oxygen uptake was measured at the same time that the rate of synthesis of ammonia and of keto acid was determined. The ratio, oxygen used:ammonia formed:keto acid formed, proved to be close to 1:2:2, as it should be if the reaction takes place as suggested in Neubauer's equation.

It will be recalled that in the chapter dealing with enzymes, the reaction catalyzed by D-amino acid oxidase was examined as an example of an oxidative enzyme action in which a coenzyme is concerned. It was shown there that the oxidative deamination of a D-amino acid takes place in two steps. There is first an enzymic dehydrogenation of the acid to form an imino acid, followed by a nonenzymic reaction in which the imino acid reacts with water to form ammonia and the corresponding keto acid.⁷



The primary hydrogen acceptor is the flavinadenine dinucleotide, which in turn passes the hydrogen on to molecular oxygen with formation of

⁶ Dr. Hans Adolph Krebs (1900–) began his research career in Germany and published the now classical papers on deamination from the University of Freiburg. About 1935 he went to England, and is now Professor of Biochemistry at the University of Sheffield. In 1953 he shared with Fritz Lipmann the Nobel Prize in Medicine.

⁷ In the equation on this page, FAD stands for flavinadenine dinucleotide, the compound which can be reversibly reduced and oxidized by addition or loss of two hydrogens, and so functions as an oxidizing coenzyme.

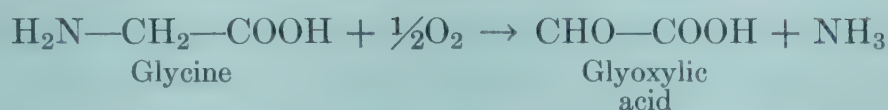
hydrogen peroxide. Since hydrogen peroxide in any great quantity is toxic, one must assume either that in living tissue the hydrogen is passed on to some other acceptor than molecular oxygen, or that there is enough catalase present to degrade the peroxide as fast as it is formed.

If the above mechanism holds for the system which catalyzes the metabolism of the natural acids, it would seem that the story of the deamination of amino acids is complete and satisfactory. It presents, however, certain anomalous features. Mammalian kidney oxidizes the unnatural amino acids, with which it normally never has to deal, ten to twenty times as fast as it attacks the natural ones. It is hard to understand why there should be available any enzyme concerned with the metabolism of acids which the organism never meets naturally. It is still more difficult to see why this enzyme should be the more active and stable one. Its greater stability led to its prior separation from the cells, at a time when the enzyme concerned with the natural acids was either completely refractory to extraction or was destroyed in the process. Some years later, Green⁸ and his colleagues obtained from rat kidneys an L-amino acid oxidase. This proved to be also a conjugated protein, but the prosthetic group of this L-oxidase is riboflavin monophosphate instead of the flavinadenine dinucleotide which functions with the D-enzyme. Experiments have shown that this L-oxidase can act upon more than half of the naturally occurring amino acids, and that the products are again keto acids, ammonia, and peroxide. However, the original hope that this enzyme would account adequately for the deamination of the natural acids has been disappointed on two counts. In the first place it is distributed far too narrowly to be quantitatively significant in animal metabolism, being low or absent in cat, dog, guinea pig, rabbit, ox, or sheep tissues. Furthermore, even in purified form its activity is strikingly low. This, of course, agrees with Krebs' original observation that kidney deaminates the unnatural acids far more rapidly than the natural ones. Up to the present no general amino acid oxidases have been found in the tissues of higher plants, but they have been obtained from several different microorganisms.

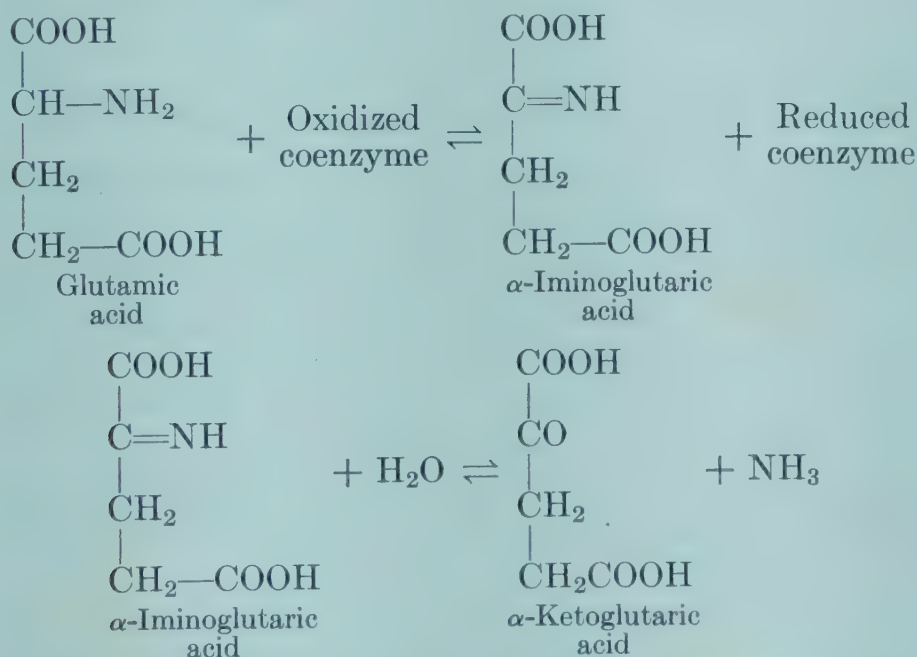
Besides the D- and the L-amino acid oxidases which attack a number of different acids, there are a few enzymes concerned specifically with the metabolism of single acids. There is a *glycine dehydrogenase*⁹ which initiates the deamination of glycine, with formation of glyoxylic acid, and an *L-glutamic dehydrogenase* which transforms this acid into the corre-

⁸ David E. Green (1910-) is an outstanding figure among the younger enzyme chemists. His specialty grew out of his graduate work at the Sir William Dunn Institute of Biochemistry, at Cambridge, England. He is now Professor of Biochemistry at the Institute for Enzyme Research of the University of Wisconsin.

⁹ The enzymes which catalyze removal of hydrogen are more correctly known as dehydrogenases, though the name oxidase is often used, as it is for the amino acid oxidases.



sponding keto acid. The latter enzyme is especially interesting because there is a good deal of evidence that glutamic acid has a central and significant place in the metabolism of amino acids. This was indicated, for example, in the Schoenheimer experiments by the fact that isotopic nitrogen was always found in higher concentration in glutamic acid than in the other amino acids. Furthermore the oxidative deamination of glutamate is reversible and this reversed reaction is believed to represent one main pathway by which ammonia is incorporated in amino acids. The enzyme, like others involved in oxidative reactions, is a conjugated protein with a prosthetic group which can be reversibly oxidized and reduced. The hydrogens which are first accepted by this coenzyme presumably find their way ultimately to molecular oxygen, as do the hydrogens donated to FAD by other amino acids.



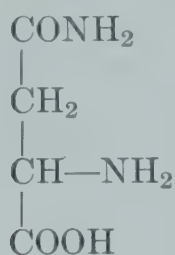
Reversal of the series as written would result in a synthesis of glutamic acid from α -ketoglutaric acid and ammonia. The enzyme which catalyzes this particular reaction has been found both in animal tissues and in certain of the higher plants.

But even with the addition of the enzymes which catalyze deamination of specific amino acids, the roster of those which must be involved in the metabolism of the natural acids is obviously incomplete. The present situation has been summed up by Cohen¹⁰ as follows: "The student . . . is presented with the anomalous situation of having comparatively de-

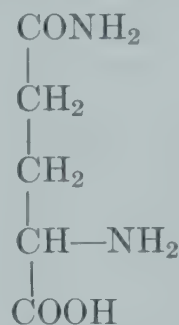
¹⁰ Dr. Philip P. Cohen (1908-) is Professor of Physiological Chemistry at the University of Wisconsin Medical School. His work is chiefly in the field of intermediate metabolism of proteins and amino acids.

tailed information concerning enzymes whose physiological function is of questionable significance and being virtually ignorant of those enzymes which might be expected to play a more important role."

Although specific enzymes are largely unidentified, it is still assumed that the reaction mechanism indicated above for the unnatural acids also holds good for the natural ones. If this is true, the end of the deamination reaction leaves the tissue possessed of keto acids and ammonia. In animals much of the ammonia is transformed in the liver into urea and excreted, though part of it is probably stored as glutamine, the half amide of glutamic acid.



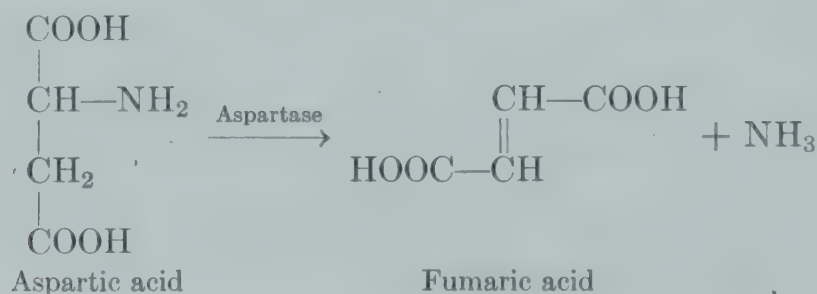
Asparagine



Glutamine

Glutamine is known to be formed in liver and in nervous tissue and both amides are present in relatively high concentration in plant seedlings. This may mean that this is a detoxication mechanism, intended to remove ammonia, or that the amides constitute a reserve supply of ammonia. The latter seems the more likely as the enzymes glutaminase and asparaginase which catalyze the reversible hydrolysis of the amides have been found widely distributed in organic nature. Thus a certain amount of ammonia can be held in reserve in a nontoxic form and liberated by enzyme action when and where needed. A further use for glutamine will be indicated very shortly.

Other Deamination Mechanisms. Although oxidative deamination is probably the principal reaction by which plant and animal cells remove nitrogen from amino acids, other pathways are known. One of these involves a simultaneous deamination and desaturation. For example, some bacteria as well as a number of plant tissues yield an enzyme *aspartase* which catalyzes the transformation of aspartic acid to fumaric acid and ammonia.



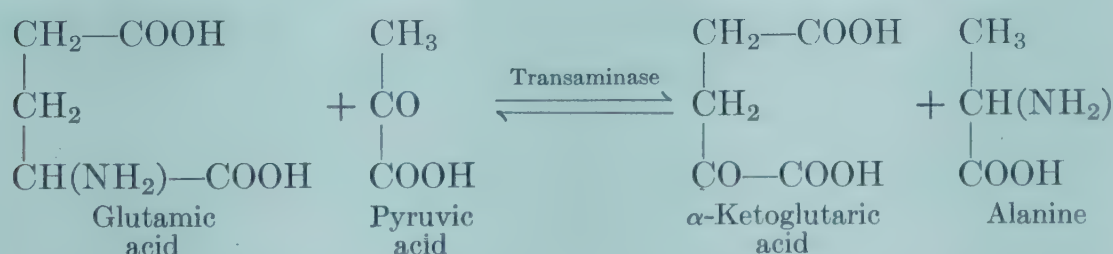
Still another deamination mechanism has been found in anaerobic bacteria. They remove amino groups reductively, forming ammonia and the corresponding fatty acid. The hydrogens which are used must come more or less directly from some other metabolite.



TRANSAMINATION

In most plant and animal tissues the main organic products of deamination are the keto acids, which then undergo further transformation. They may be oxidized completely to carbon dioxide and water. They may be used for synthesis of carbohydrate or of fatty metabolites. Or they may acquire new amino groups and so again become members of the metabolic pool of amino acids. This last reaction is known as *transamination*.

In 1937 Braunstein and Kritzmann reported from the Institute for Experimental Medicine in Moscow their discovery of an enzymic reaction by which certain keto acids may be transformed into amino acids. They showed that many different plant and animal tissues contain an enzyme which catalyzes a reaction between a keto acid and an amino acid, in the course of which the amino acid donates its basic group to the keto acid. In the original experiments glutamic acid reacted with pyruvic acid to form alanine, itself becoming α -ketoglutaric acid. Because of this interchange the authors referred to the reaction as an *umaminierung*, which has been translated *transamination*.



The discoverers assumed that the transamination reaction had a very broad application and that any amino acid could thus transfer its amino group to any one of a wide variety of keto acids. For some years this was questioned, as the first transaminases to be isolated proved to be very specific and to catalyze transfer of amino groups from glutamate to just two keto acids. More recent work however has fully established the general scope of the reaction. Transaminases have been found in bacteria, in higher plants, and in various animal tissues. They catalyze the transfer of amino groups from nearly all the natural amino acids to a number of different keto acids. Those compounds which are known to be donors and acceptors of amino groups are listed in Table 11-VI. Thus the transamination reaction offers a simple explanation of that lability of amino groups which came out so strikingly in the isotope experiments with ani-

TABLE 11-VI. ACIDS KNOWN TO PARTICIPATE IN TRANSAMINATIONS

Donor Acids	Acceptor Acids ^a
Leucine	Pyruvic acid
Phenylalanine	α -Ketoglutaric acid
Methionine	$\text{C}_6\text{H}_5\text{—CH}_2\text{—CO—COOH}$
Histidine	$\text{HO—C}_6\text{H}_4\text{—CH}_2\text{—CO—COOH}$
Tyrosine	$\text{HOOC—CH}_2\text{—CO—COOH}$
Ornithine	
Arginine	
Valine	
Aspartic acid	
Glycine	
Tryptophan	
Ammonium glutamate	
Glutamate	
Serine	
Alanine	

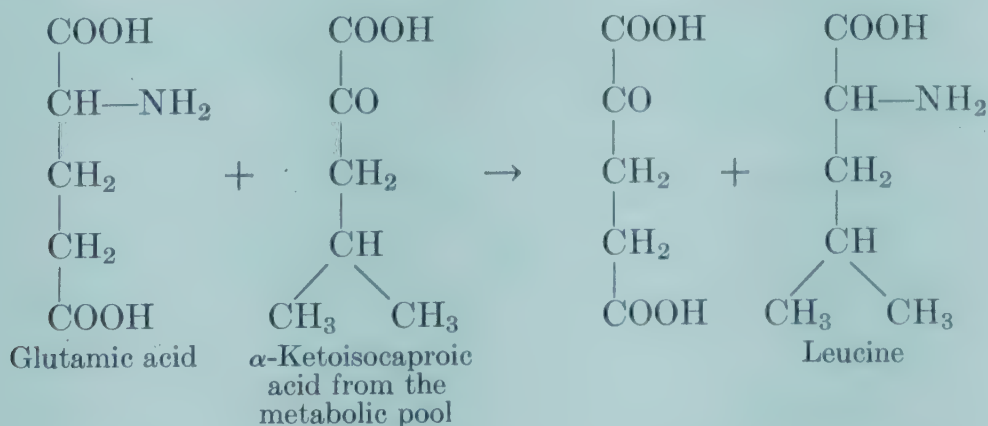
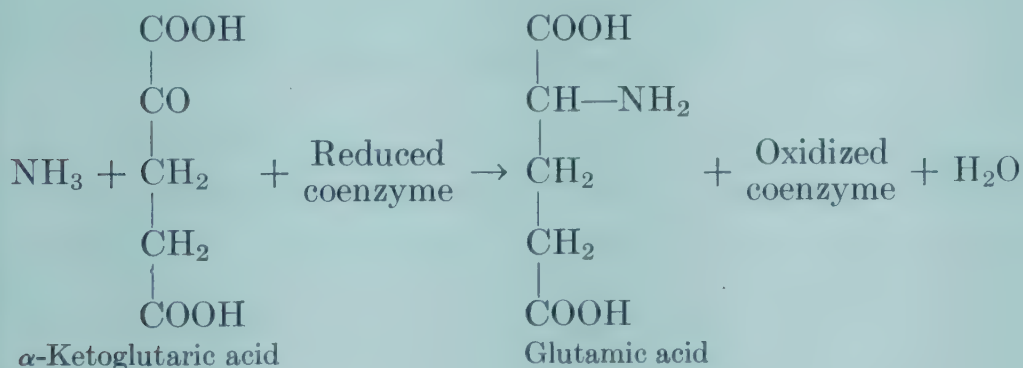
^a Not all donors have been tested with all acceptors.

mals. Deamination transforms the amino acids into keto acids. Many of these keto acids then accept new amino groups from other amino acids in the vicinity and in this way any isotopic nitrogen administered in an amino acid is soon distributed at random among most of the members of the entire pool. It will be remembered that lysine was the one amino acid which did not acquire labeled nitrogen in the Schoenheimer experiments. Threonine is the only other acid which has since proved to share this inertness. This is now taken to mean that for some unknown reason these two acids do not take part in the transamination reactions which otherwise are so characteristic of this group of compounds.

Quite recently it has been found that it is not only the free acids which undergo transamination. Liver enzymes bring about the transfer of the α -amino group of glutamine to a number of different keto acids, with simultaneous hydrolysis of the amide itself to a free keto acid. The properties of the enzyme (or enzymes) involved show that it is quite distinct from the transaminases which act on the free amino acids. Here then is a definite function for glutamine in animal metabolism. It is likely that both glutamine and asparagine perform a like function in plants.

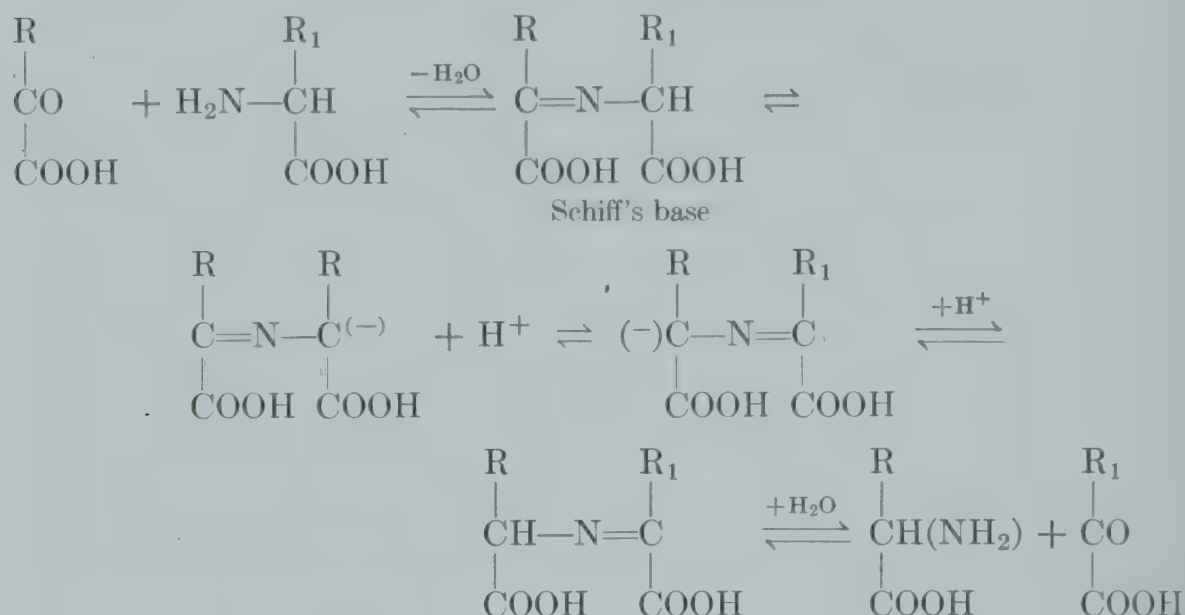
The significance of the transamination reaction lies in the fact that keto acids arise not only in deamination of amino acids but also in the course of carbohydrate breakdown in plants, animals, and microorganisms. Furthermore it is very likely that several of these compounds are formed also in the course of photosynthesis. Under these circumstances the synthesis of amino acids from keto acids by transamination is not merely a kind of Grand March, with amino acids changing partners all down the line, but neither increasing nor decreasing in total amount. The process is on the

other hand a true synthesis in the sense that some entirely new amino acids will arise. In animals the nitrogen for these acids is largely obtained from preëxisting amino acids it is true, but the synthesis may well provide an uncommon acid at the expense of such a ubiquitous one as glycine or alanine. In plants the precursors of the amino groups are ammonium salts and nitrates of the soil, the latter reduced by the plant to the ammonia level. Consider then a system in which glutamine is available for amino acid synthesis. This compound is chosen partly because it provides two amino groups, and partly because glutamic acid is the one amino acid which has been synthesized enzymically by interaction of free ammonia and a keto acid. Its activity as a donor in the transamination reaction suggests that this may be the pathway by which free ammonia is carried into most of the amino acids. Thus if the first reaction of glutamine is the transamination referred to above, there will result a new amino acid, free ammonia, and a molecule of α -ketoglutarate. But the ammonia and the keto acid can now react with each other to form glutamic acid, in the reversal of the classical deamination reaction (see p. 353) and thus the second amino group becomes available for transfer. The chart indicates the reactions by which a molecule of leucine might be built up from α -ketoisocaproic acid and ammonia using α -ketoglutarate first as an acceptor and then as a donor of an amino group.



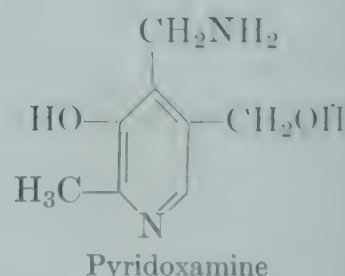
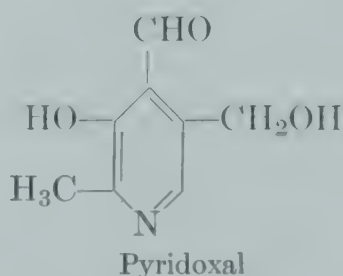
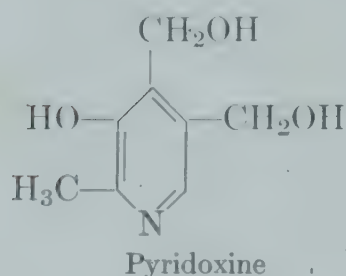
Mechanism of Transamination. Reactions similar to enzymic transaminations may be brought about at elevated temperatures without the use of a

biocatalyst. In such reactions there is evidence that the transfer takes place through the intermediate formation of a compound of the type known as a *Schiff's base*. Braunstein and Kritzmann suggested a similar mechanism for the enzymic reaction.



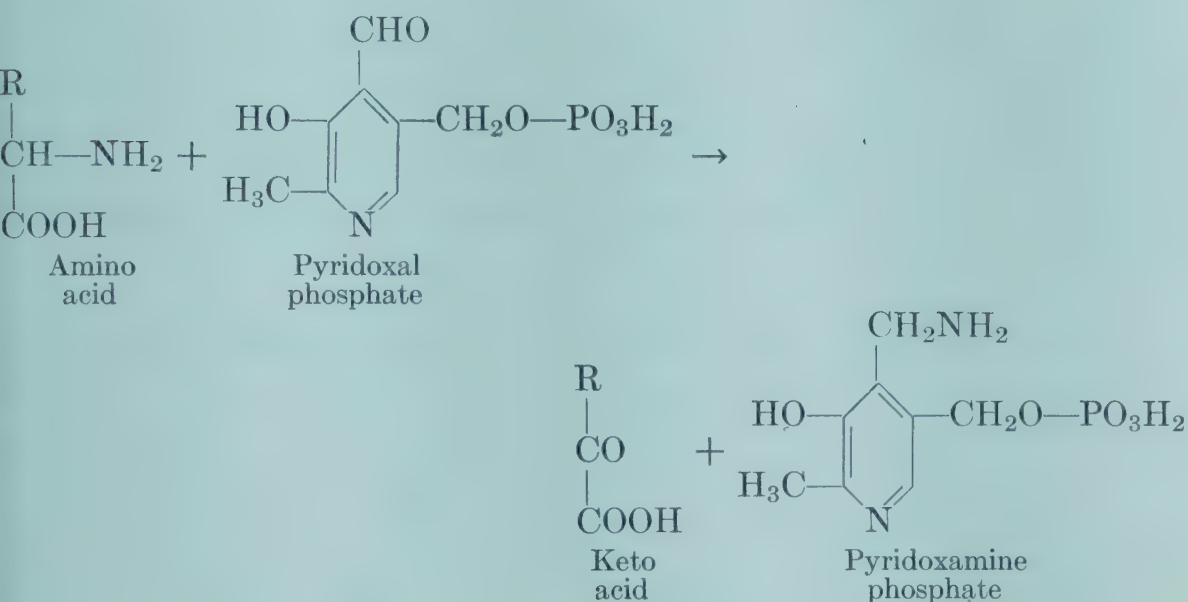
According to this scheme there is first formed an addition compound, which by the two-stage shift of a proton undergoes an intramolecular rearrangement which moves the double bond from one side of the nitrogen to the other. Hydrolysis of this rearranged addition product yields the new keto and amino acids.

This mechanism was accepted until it was shown that most of the transaminases occur as conjugated proteins. The obligatory coenzyme proves to be a phosphorylated derivative of one of the B vitamins. A number of closely related pyridine derivatives, capable of preventing a certain type of rat dermatitis, are now grouped together as the "B₆ vitamins," though B₆ was at one time believed to be a single substance, pyridoxine. The formulas below show the relationship to pyridoxine of the two compounds, pyridoxal and pyridoxamine, which in phosphorylated form act as coenzymes of transamination.

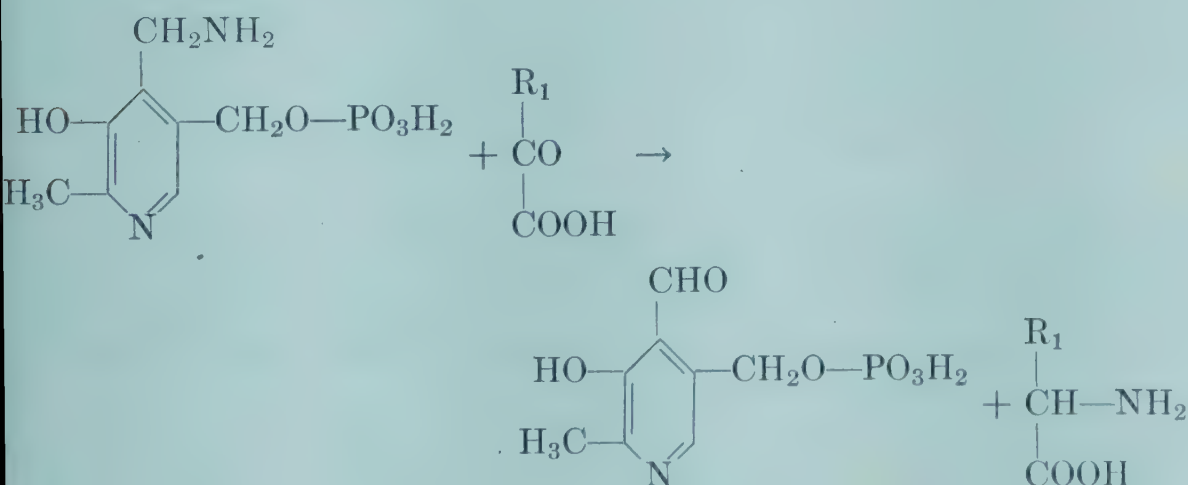


It now appears that the transamination reaction takes place in two steps, in the course of which the coenzyme transfers an amino group by being

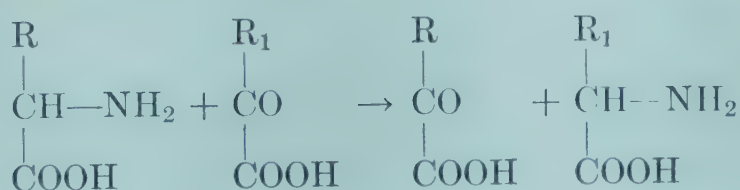
alternately aminated and deaminated. For example, the first step may be transamination of pyridoxal phosphate with an α -amino acid, probably through the intermediate formation of an addition compound of the Schiff's base type as shown above. This yields a keto acid and pyridoxamine phosphate.



The newly formed pyridoxamine phosphate then takes part in a similar reaction with a keto acid, regenerating the original pyridoxal phosphate and forming a new amino acid.

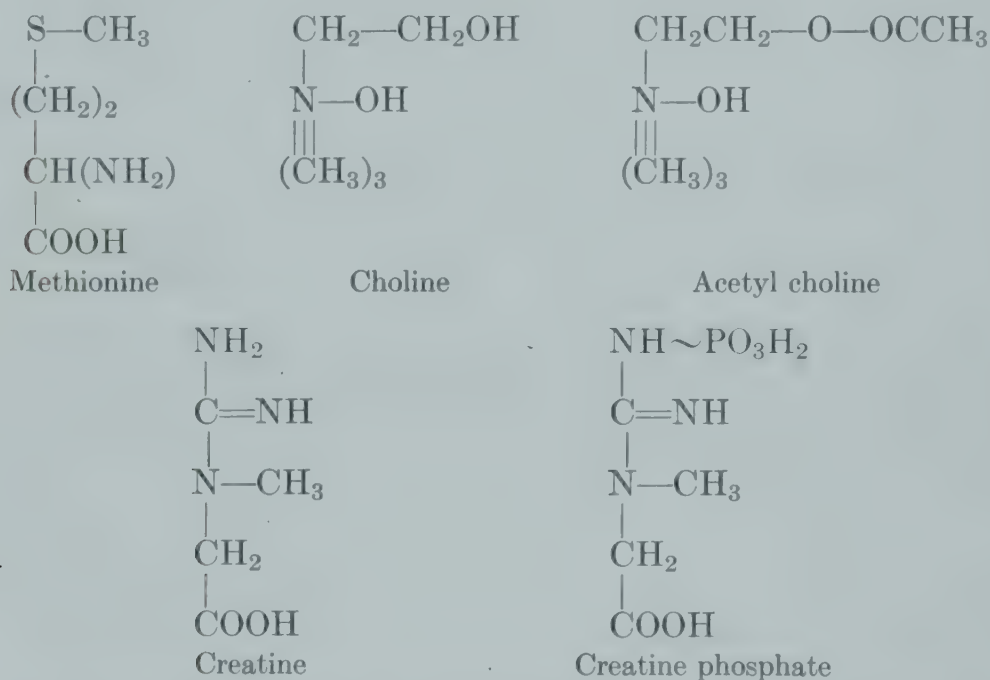


The net result may then be summed up in the typical transamination equation.



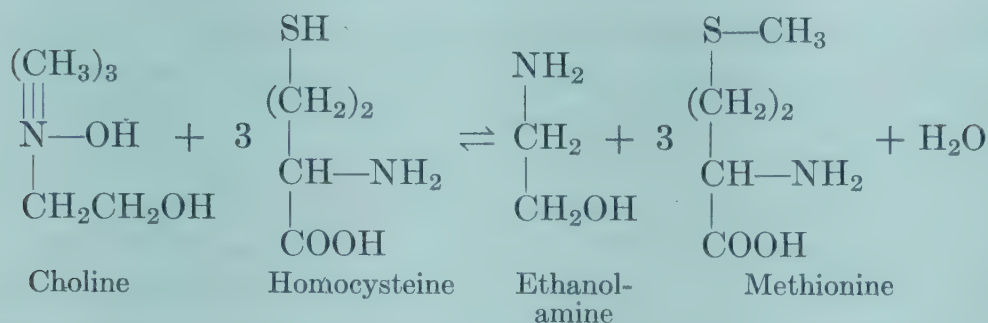
TRANSMETHYLATION

Although most of the amino acids are promptly deaminated, a certain few are involved, as amino acids, in other metabolic transformations. One such transformation is the one known as *transmethylation*. There are a number of nitrogenous compounds of fundamental importance to the living organism which contain one or more methyl groups. These include *methionine* which is one of the indispensable amino acids; *choline* which is itself a constituent of some of the phospholipids and which in the form of acetyl choline plays a part in transmission of nerve impulses; and *creatine* which, as creatine phosphate, serves a unique function in carbohydrate metabolism in muscle.



The three parent substances, methionine, choline, and creatine, are all interrelated through their participation in the transmethylation reaction. It was believed until very recently that the animal body is incapable of generating methyl groups and is therefore dependent upon a supply of methionine or choline in the food to provide them. Homocysteine¹¹ ($\text{SH}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$) is a satisfactory substitute for the so-called "indispensable" methionine if the diet includes an adequate supply of choline. Methyl groups can be transferred from the nitrogen atom of choline to the sulfur atom of the homocysteine, thus synthesizing methionine and ethanolamine. It will be recalled that this latter substance is found in the body combined with glycerol, phosphoric acid, and fatty acids in phosphatidyl ethanolamine.

¹¹ The prefix homo- is used to indicate that a compound is the next higher homologue of the more common substance. Thus homocysteine has one more carbon than cysteine and homoserine is $\text{HOCH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$.

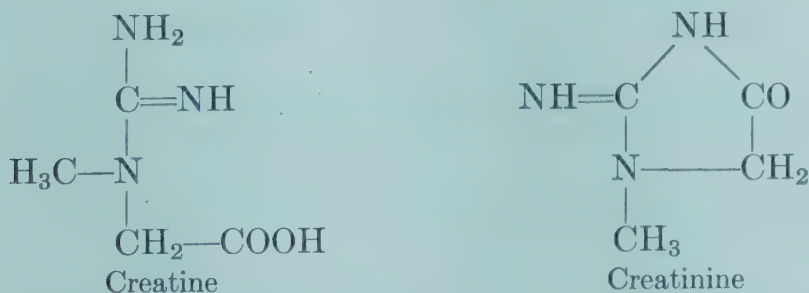


This transmethylation reaction is reversible, and so when conditions require it, dietary methionine is capable of furnishing methyl groups for the synthesis of choline. Experimental proof of reversibility has been obtained by the use of deuterium. Methionine was synthesized containing deuterium in the methyl group. After feeding the labeled methionine to animals, choline was isolated from their tissues. This proved to contain enough deuterium to establish clearly the transfer of the marked methyl groups. We shall see in the next section that a similar transfer of intact methyl groups from methionine is involved in the synthesis of creatine.

Although animals undoubtedly do use the transmethylation reaction, it has been shown in very recent years that they are not entirely dependent upon an adequate dietary supply of labile methyl groups. Work with labeled molecules has shown that the carbon of formate can be used by liver slices in the synthesis of methyl groups, and that deuterium of injected heavy water is also incorporated into these groups, which must therefore be synthesized in the body. It therefore seems likely that animals share with plants the ability to synthesize methyl groups from water and simple carbon compounds, but that they also make use of transmethylation in maintaining a proper balance in the amounts of the various methylated compounds in the cells.

CREATINE AND CREATININE

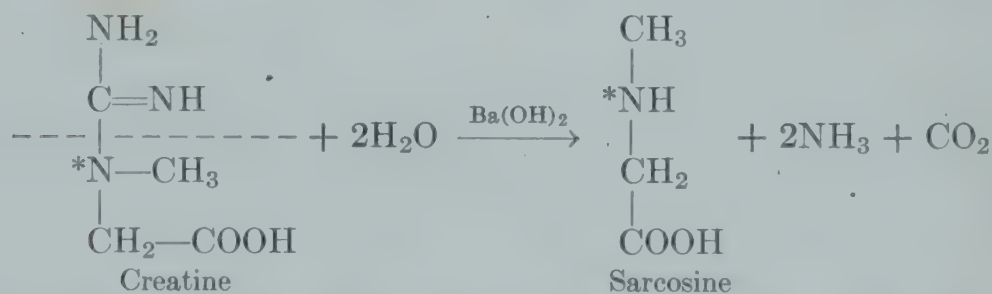
For many years an outstanding biochemical problem was the relation between body creatine and the nearly constant amount of its dehydration product, creatinine, found in the urine.



It had been found that feeding large amounts of creatine did not lead to an immediate increase in creatinine excretion. From this it had been argued that creatine was not the source of urinary creatinine at all. It is

now known that when creatine is fed in excess the body is capable of storing it, probably as creatine phosphate, up to a certain saturation point. Only after this point is reached does the usual level (1.5–2 g. per day in man) of creatinine excretion gradually begin to be exceeded. That this delayed excretory product actually arises from body creatine has been shown by experiments with N^{15} . Creatine, marked with N^{15} , was fed for a considerable time, and then for a time the diet was maintained entirely free of creatine. During the latter period the concentration of the isotope in the creatinine excreted was the same as that in the tissue creatine, thus indicating the transformation of the latter into creatinine. This dehydration is apparently irreversible in the body, for when isotopically labeled creatinine is added to the diet, none of the isotope appears in the body creatine. Dietary creatinine is excreted as such.

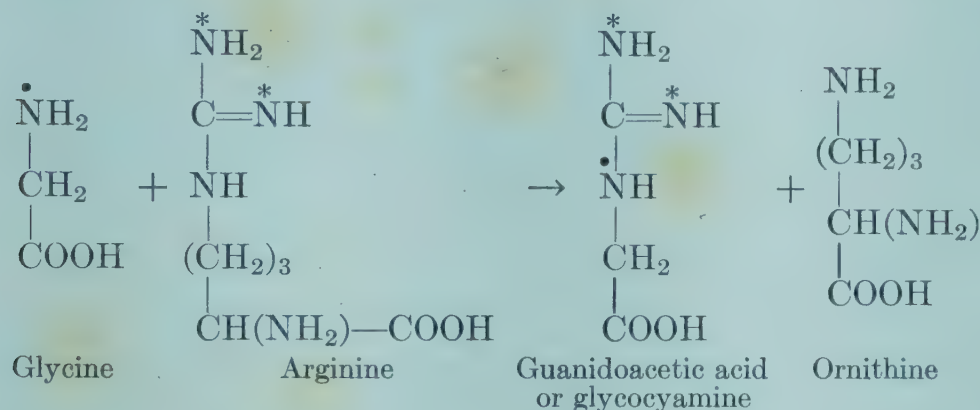
It has long been clear that the body is capable of synthesizing whatever creatine it needs. The only possible source of preformed creatine in a diet is meat, and no ordinary amount of meat could provide enough creatine to account for the normal daily excretion of creatinine. Furthermore, creatinine continues to be excreted at this level even when the diet contains no meat whatever. The search for the precursors of creatine was a long one, but yielded only equivocal results until the advent of isotopic tracers. Following the administration of N^{15} in the α -position in several different amino acids, as well as in urea and ammonia, the composition of the tissue creatine was investigated. Of the amino acids investigated only glycine gave rise to muscle creatine containing enough of the isotope to indicate a true precursor. To determine the position taken by the N^{15} in the creatine molecule, it was degraded by boiling with alkali, which brings about the following hydrolysis:



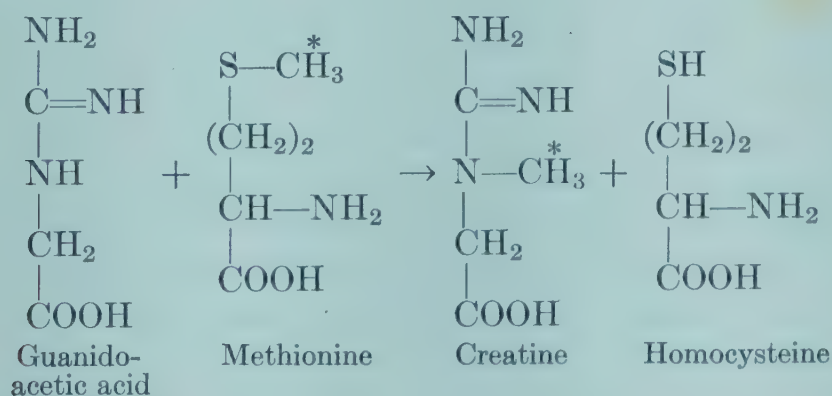
As indicated by the starred atoms in the formulas, the tracer was found almost entirely in the sarcosine. This showed that glycine contributes to creatine its own α -nitrogen, but not nitrogen for the synthesis of the amidine group ($\text{NH}_2\text{---C}=\text{NH}$).

Various possible precursors of the amidine group were then investigated, and of these only guanidoacetic acid ($\text{NH}_2\text{---C}(=\text{NH})\text{---NH---CH}_2\text{COOH}$) or glycoylamine proved to contribute to the synthesis of creatine. It therefore seemed likely that glycine is first transformed into guanidoacetic

acid, and that transfer of a methyl group then completes the synthesis. This reaction sequence was proved by experiments in which arginine, labeled in its amidine group, was fed to rats. Again the creatine was isolated and hydrolyzed, but this time the isotope was nearly all found in the ammonia set free. It had therefore been almost exclusively in the amidine group of the creatine. In the following equations the marked nitrogens in the two compounds are differently labeled to indicate the source of each nitrogen in the creatine precursor. Thus two carbons and one nitrogen of creatine come from glycine and the amidine group from arginine.



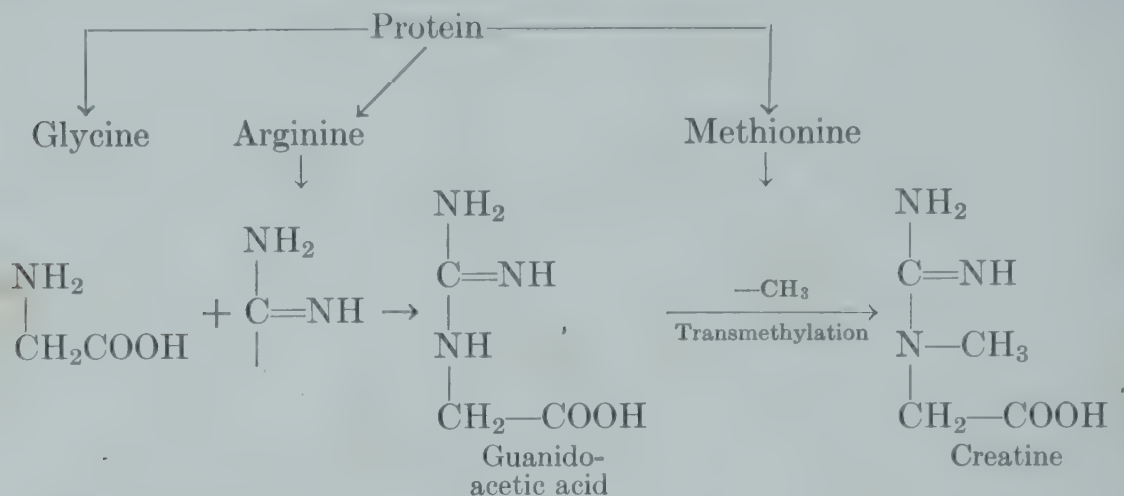
It remained for du Vigneaud¹² and his colleagues to show that a third amino acid, methionine, is the source of the methyl group. Methionine marked with deuterium in its methyl group (trideutero-methionine) was fed to rats and subsequently creatine was isolated from their muscles. It proved to contain a methyl group clearly marked with deuterium, thus proving that transmethylation had been responsible for providing the methyl group.



Thus here again we find evidence of the importance of the transmethylation reaction. It seems that the indispensability of methionine resides

¹² Vincent du Vigneaud (1901-) is Professor of Biochemistry at the Cornell University Medical College in New York. His biochemical research has reflected his interest in classical organic chemistry, and he and his students have made fundamental contributions to our knowledge of amino acid chemistry and metabolism, sulfur metabolism, and the chemistry of biotin and penicillin.

largely in its ability to furnish labile methyl groups. The following chart, adapted from Schoenheimer's book, indicates the way in which creatine is derived from three different amino acids.



USES OF THE AMINO ACIDS

Obviously the chief function of the amino acids, whether derived directly from the foods or formed in the organism, is the synthesis of proteins. To meet the needs of a growing organism new structural proteins as well as enzymes must be elaborated. But the work of Schoenheimer has shown that even when there is no question of growth, the tissue proteins are subject to a continuous process of regeneration from the metabolic pool of amino acids. The mechanism of this interchange is not at present understood. As might have been expected, it takes place only so long as the system includes some source of energy, for formation of peptide bonds is an endergonic process. This energy may be provided as it is in a living organism by oxidation, or the energy needs of an isolated enzyme system may be met by addition of adenosine triphosphate (ATP) with its two high energy bonds. We shall find as we go on that the energy of these bonds is often used by living cells as an immediate energy source.

Glycogenesis and Ketogenesis. A normal diet provides more amino acids than can possibly be used in protein synthesis. The keto acids derived from some of these therefore become available to meet the more general energy needs of the organism. At one time biochemists were much concerned to decide whether or not these extra acids were transformed in the body into either of the other primary foodstuffs. The answers were obtained in experiments with animals rendered experimentally diabetic, so that instead of oxidizing glucose they excreted it in the urine. It was found that administration of certain of the amino acids to such animals led to a prompt increase in glucose excretion. Furthermore when these same acids were fed to animals whose glycogen stores had been depleted by starvation, the deposition of glycogen in the liver again indicated trans-

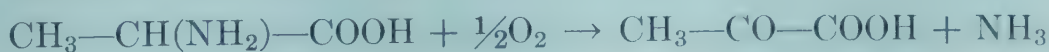
formation of amino acids into carbohydrates. Recent results with amino acids labeled with isotopic carbon are even more definite. The incorporation of the isotope in liver glycogen proves conclusively that the carbons of amino acids can be used in the body for carbohydrate synthesis. The amino acids which can be used in this way are said to be *glycogenic*.

In cases of severe diabetes it is not only the carbohydrate metabolism which is deranged. Failure of the organism to metabolize normally the fats of the diet gives rise in the blood and urine to a group of compounds known as "ketone bodies" of which the most fundamental is acetoacetic acid, $\text{CH}_3\text{—CO—CH}_2\text{—COOH}$. Administration of a few of the individual amino acids to diabetic animals is followed by an increased excretion of ketone bodies, thus linking their metabolism to that of the fatty acids. Furthermore, perfusion of a liver with these same amino acids leads to formation of acetoacetate in the perfusion fluid, and isotopically labeled acids give rise to labeled acetoacetate. The amino acids which can be transformed in the organism to acetoacetate are said to be *ketogenic*. The acids in the two groups are listed in Table 11-VII. It should be noted that the two lists do not include all the natural amino acids.

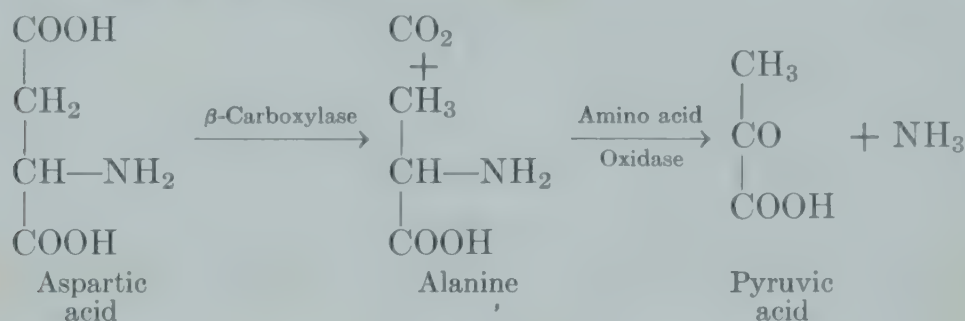
TABLE 11-VII. METABOLIC USES OF AMINO ACIDS

Glycogenic Amino Acids	Ketogenic Amino Acids
Glycine	Leucine
Alanine	Tyrosine
Serine	Phenylalanine
Cysteine	
Aspartic acid	
Glutamic acid	
Proline	
Oxyproline	
Ornithine	
Arginine	
Threonine	

The Metabolic Pool. Although it is still of interest to know how the body uses the excess molecules of keto acids formed by deamination, the formation of glycogen or glucose from them is not the direct "transformation" that was once envisaged. We shall see when we come to the metabolic reactions of fats and carbohydrates that certain compounds are common to several metabolic paths. Thus a fundamental product of carbohydrate metabolism is pyruvic acid, $\text{CH}_3\text{—CO—COOH}$, and it has been shown that a number of different amino acids also yield pyruvate in the presence of tissue enzymes. Alanine is the simplest example, forming pyruvic acid by simple oxidative deamination.



But other acids also act as pyruvate precursors. Aspartic acid, for example, is decarboxylated under the influence of a β -carboxylase found in liver, yielding carbon dioxide and alanine, which latter forms pyruvate when it is deaminated.



It should be noted that the type of decarboxylation undergone by aspartic acid is not the reaction generally expected of an α -amino acid. These compounds normally lose the α -carboxyl group forming primary amines (see p. 160).

By a more complex series of transformations glutamic and other amino acids also yield pyruvic acid. In general, then, certain of the amino acids, of which these three will serve as examples, add to the general metabolic pool of pyruvate. Since all of the reactions by which pyruvate is formed from glucose are reversible, the carbohydrate formed from labeled amino acids may result from a simple reversal of the reactions of glucose breakdown, labeled pyruvate leading to formation of labeled glucose and this in turn condensing to form labeled glycogen.

Active Acetate: An alternative use of pyruvate will be considered in detail in the final chapter of this book, but may be briefly indicated here. Pyruvic acid loses carbon dioxide readily in an oxidative reaction which leaves its other two carbons in a form which was referred to for many years as "active acetate." What is actually formed is an acetyl derivative of a substance known as coenzyme A. This derivative, abbreviated to "acetyl CoA," in which the acetyl group replaces a hydrogen of the original coenzyme, is capable of entering a wide variety of synthetic processes. In these the two carbons of the acetyl group are used and the coenzyme is set free to transport another acetyl group. Among the compounds known to be formed by this two-carbon fragment is acetoacetic acid which results from condensation of two molecules of acetyl CoA.



This means, then, that the amino acids, or the corresponding keto acids, contribute to the metabolic pool various small molecules which there become indistinguishable from similar compounds derived from fats or carbohydrates. The further transformation of these small molecules may lead to synthesis of fatty acids, of acetoacetate, or of a number of other com-

pounds including glucose. Thus the dynamic equilibrium established in living cells includes not only the proteins and their constituent amino acids but all the primary foodstuffs and their metabolic derivatives.

Active Formate: Evidence is beginning to emerge that the body uses for its synthetic purposes not only an "active" two-carbon compound, now identified as acetyl CoA, but a one-carbon intermediate which appears to be a derivative of formate. The concept of active formate as a key metabolic intermediate depended originally upon evidence that single labeled carbon atoms of various amino acids are used in synthesis both by animals and by microorganisms. Later it was shown that free formate could often be substituted for the original compound, and the number of syntheses in which formate is now known to be involved is a long one. To take a single example, there are several biosyntheses to which glycine contributes only one of its two carbon atoms. This is believed to result from a preliminary rupturing of the carbon chain to yield formate or some closely related compound which then goes on to take its place in the synthetic process. It is presumably in this way that glycine contributes to the synthesis of methyl groups, referred to above. When rats are fed either labeled formate or glycine labeled in its α -carbon ($\text{NH}_2\text{—C}^{14}\text{H}_2\text{—COOH}$) the isotope is found in the methyl groups of choline. This is interpreted to mean that formate is an intermediate between glycine and the methyl groups. On this same type of evidence several other amino acids are also believed to contribute to the supply of a one-carbon intermediate which is used by the cells in a variety of syntheses.

In summary, the amino acids are needed primarily for protein synthesis which involves a highly labile dynamic equilibrium among amino acids, keto acids, and cell proteins. In the course of establishing this equilibrium keto acids are formed and ammonia is set free. A major part of the latter compound is excreted as urea though smaller amounts are probably conserved as the half amides of the dibasic amino acids and some of it may be returned to the amino acids by way of glutamic acid. The keto acids which are not promptly reaminated may be completely oxidized to carbon dioxide and water or they may be degraded into small molecules which can be caught up in the metabolic machinery and used in synthesis. Of outstanding significance among these small molecules are pyruvate, acetate, and formate.

EXCRETION OF NITROGEN

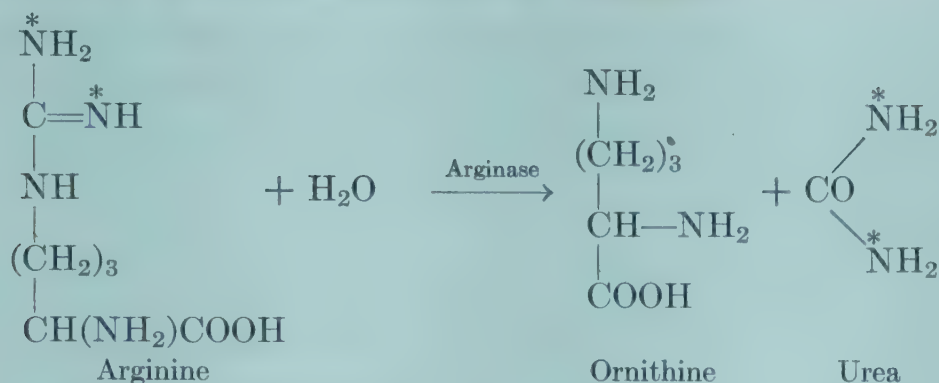
Since plants manufacture their own amino acids they escape the necessity for excreting excess nitrogen. In animals the main nitrogenous excretory products are urea, ammonia, creatinine, and uric acid. We have already seen that creatinine is formed by dehydration of body creatine, preparatory to its excretion. Although this reaction is reversible in the laboratory, it is one of the small number of irreversible metabolic reactions.

The amount of creatinine excreted is remarkably independent of the diet, and varies within very narrow limits. A normal man excretes 1.5–2.0 g. per day. Presumably the body synthesizes creatine at this rate. Creatinine nitrogen makes up about 3–7 per cent of the total nitrogen in the urine.

The amounts of the other nitrogenous constituents excreted depend largely upon the diet. Since uric acid is the end product of purine metabolism in man, the extent of its excretion depends upon the amount of nucleoprotein ingested. On a diet which includes a high percentage of liver or of sweetbreads, both rich in nuclear material, the daily output may be as high as 2 g., while a purine-free diet may lower the uric acid output to 0.1 g. per day. In health and on an average diet, about 0.7 g. of uric acid is excreted per day, corresponding to 1–3 per cent of the total urinary nitrogen.

Urea and ammonia arise from amino acids and the amounts of these substances excreted also depend upon the protein content of the diet. In one experiment Folin found that a man on a normal protein diet excreted 31.5 g. of urea in twenty-four hours, but on a starch-cream diet only 4.72 g. "Ammonia" appears in the urine as ammonium salts of various organic and inorganic acids, hydrochloric, sulfuric, and phosphoric acids predominating normally. It was noted above that the kidney is the most active deaminating organ in the body. This ability rapidly to set free ammonia from amino acids is very necessary to an organ which must from time to time neutralize undetermined amounts of acids before excreting them. An acid-forming diet results in an increased ammonia excretion, whereas base-forming foods tend to lower the amount of ammonia excreted. Ordinarily about 2.5–6 per cent of the total urinary nitrogen is in the form of ammonium salts; urea makes up about 85 per cent of the whole. Thus the bulk of waste nitrogen in man is excreted as urea.

Urea Synthesis. The work of Bollman and his colleagues, to which reference has already been made (p. 350), proved that urea formation takes place only in the liver, and that it is preceded by the setting free of ammonia. In 1932 Krebs and Henseleit showed that the process of urea formation from ammonia is one of the complex, cyclical procedures which seem to suit the needs of living cells. Working with tissue slices they found that urea is formed by liver whenever either amino acids or ammonia are furnished in the nutrient solution. Since different amino acids are deaminated at different rates, the rate of urea formation from these precursors also varies from acid to acid. But when arginine was used with tissue slices, the amount of urea formed greatly exceeded that which could possibly have arisen by deamination of the amino acid itself. This indicated that the arginine molecules were functioning in some catalytic fashion and were probably being used repeatedly. It was already known that the liver contains an enzyme, arginase, capable of splitting arginine to form urea and the simpler diamino acid, ornithine.



Krebs found that small amounts of added ornithine exerted the same catalytic effect as did arginine. On the basis of these results the authors suggested that ammonia derived from amino acids in deamination is transformed to urea in the series of reactions shown in Figure 11.2. Ammonia and carbon dioxide reacting with ornithine form citrulline. This then condenses with a second molecule of ammonia to form arginine which in

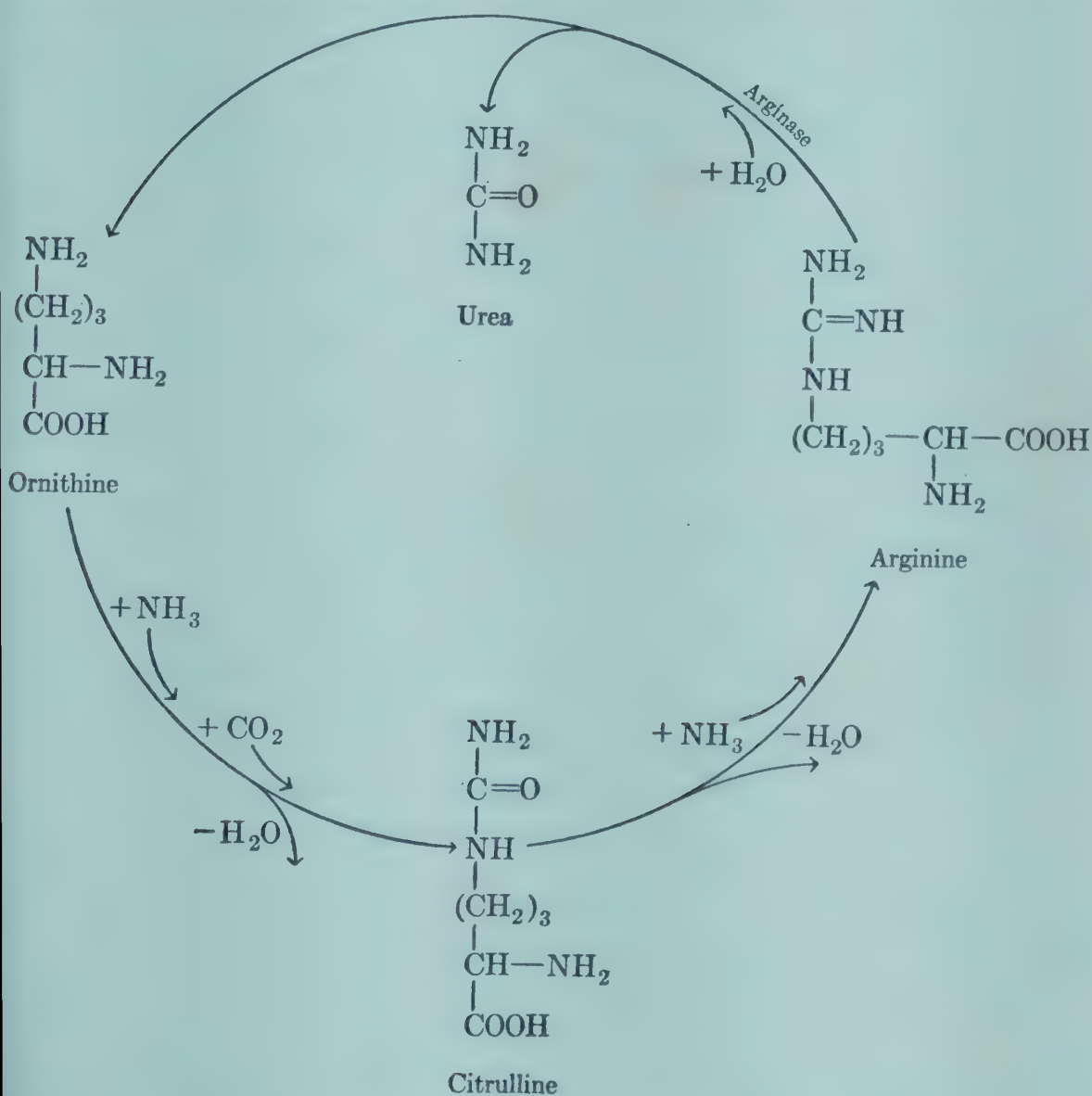


Figure 11.2. Krebs urea cycle.

hydrolysis sets free urea, and reconstitutes the ornithine. The net result is that metabolic ammonia and carbon dioxide are transformed into urea, with a regeneration of ornithine at the end of each cycle. Thus a small amount of any one of the three amino acids serves to bring about formation of large quantities of urea.

Later work with isotopic tracers has amply supported Krebs' suggested mechanism. Feeding isotopic ammonia or labeled amino acids leads to excretion of marked urea. At the same time, arginine isolated from the *body proteins* of the animals involved also proves to contain the nitrogen isotope. When this arginine is hydrolyzed by arginase, all the N^{15} is found in the urea set free, thus indicating that it had originally been part of the amidine group of the amino acid. The positions occupied by the isotopic nitrogen atoms in this experiment are starred in the equation given on page 369.

The presence in the organ proteins of arginine with nitrogen isotope in its amidine group points clearly to its having participated at some time as free amino acid in the urea cycle. The ornithine which had been formed when unlabeled body arginine was hydrolyzed, acquiring new nitrogen from the metabolic pool of ammonia, had picked up a certain number of the labeled molecules and had carried this nitrogen back with it when, as newly formed arginine, it reentered a protein molecule. Presumably many other arginine molecules did not survive to be used again in body proteins, but were promptly acted upon by arginase to set free more urea, thus accounting for the isotopically labeled urea. Further evidence for the Krebs urea cycle was obtained by administration of arginine itself, marked in its amidine group. Only a very little of the isotope found its way into liver proteins; more than 60 per cent of it was recovered as urea.

The participation of carbon dioxide in the cycle has been demonstrated both with stable C^{13} and with radioactive C^{14} . The carbon dioxide-bicarbonate buffer used in experiments with liver slices was marked with one of the carbon isotopes. Urea isolated at the end of the experiment contained a high enough concentration of the marker to indicate that the carbon dioxide of the nutrient medium had shared with metabolic carbon dioxide of the tissue slices, the function of providing the carbon atom.

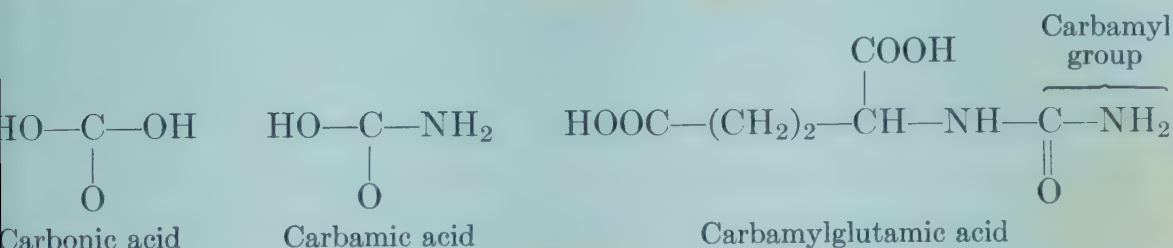
As this is being written, the Krebs-Henseleit urea cycle is in process of further analysis, and is proving far more complex than the three simple reactions originally postulated. The enzyme arginase which brings about the final hydrolysis was separated from liver cells many years ago, but until 1948 all the other enzymes of the cycle proved refractory. In order to bring about the transformation of amino acids into urea it was necessary to use liver slices with all the battery of enzymes they contain. But in the last few years the enzyme systems have been extracted from the cells and some progress has been made in separating the various component

catalysts. This has made it possible to study separately the synthesis of citrulline from ornithine and the synthesis of arginine from citrulline.

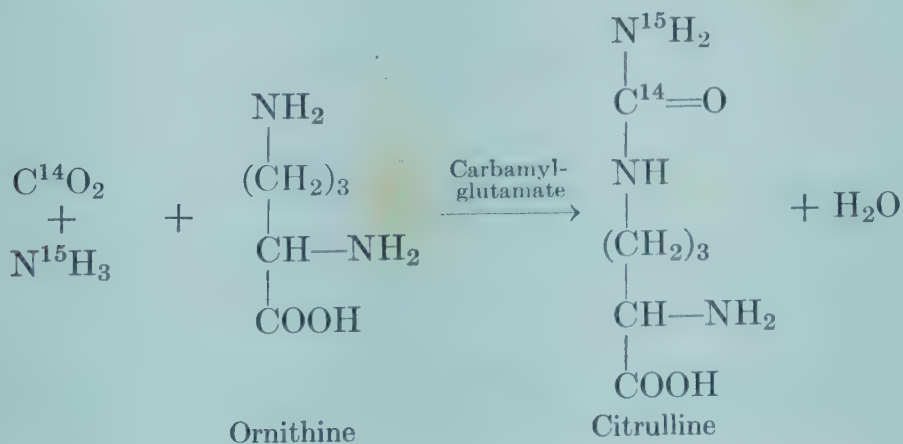
It was originally assumed that the transformation of ornithine to citrulline involved a direct reaction between ornithine and metabolic carbon dioxide and ammonia. That this is not so was first indicated by evidence that glutamic acid is essential to the system and that citrulline synthesis takes place only if some source of energy is provided. This may be done by allowing the synthesis to be coupled with an active oxidative metabolism or by providing energy preformed in the high energy bonds of ATP. Either mechanism releases energy needed for the endergonic reactions of citrulline synthesis. The part played by glutamic acid is indicated by the following observations:

1. Citrulline synthesis from ornithine is greatly accelerated if glutamate, carbon dioxide, and ammonia are incubated together for some time before the ornithine is added.

2. Of a great number of compounds examined as possible substitutes for glutamic acid, only carbamyl-L-glutamic acid is effective, and indeed this substance is very much more active than is the free acid. Carbamyl-glutamic acid is a substituted amide formed by interaction not with ammonia but with the half amide of carbonic acid which is known as carbamic acid.

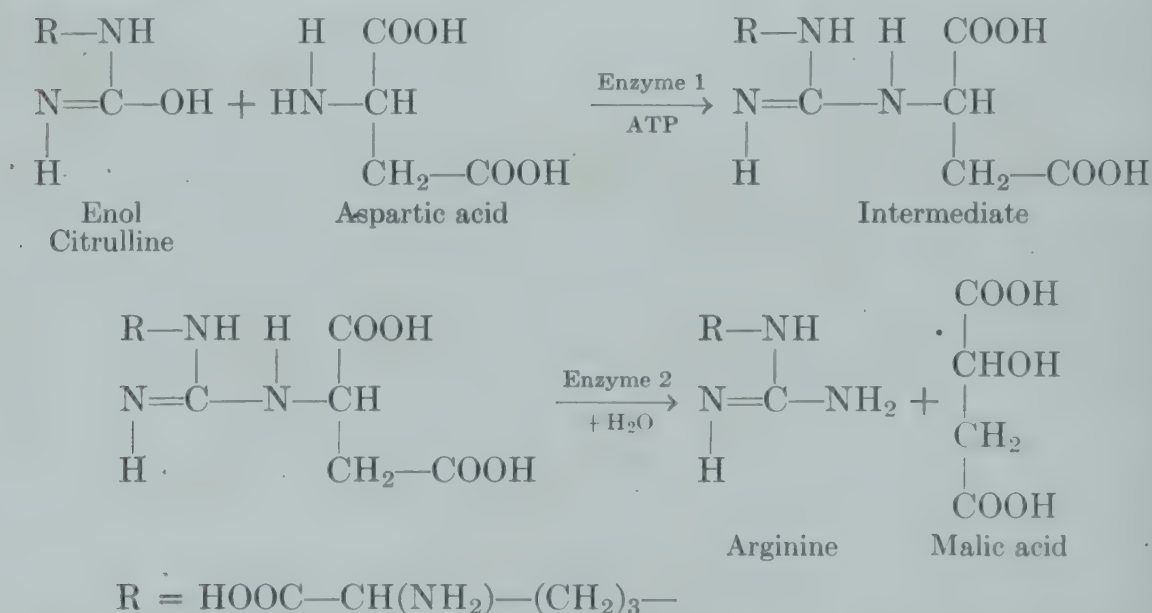


The exact role played by the carbamylglutamate is still in doubt. It does not transfer its carbamyl group directly to ornithine for it has been shown that the new carbon and nitrogen in the citrulline arise from carbon dioxide and ammonia and not from the carbamylglutamate. This was proved with isotopically labeled molecules as indicated in the formulation of the reaction.



Cohen and his colleagues, who have carried out all the recent work on citrulline synthesis, postulate the intermediate formation of a complex made up of carbamylglutamate with ammonia and carbon dioxide. Reaction of such a complex with ornithine would then form citrulline and reconstitute the carbamylglutamate. Up to the present no such complex has been isolated, and so the complete clarification of Step I in the urea cycle is still impossible.

Step II, the synthesis of arginine from citrulline, has been greatly clarified by the work of Ratner¹³ and her associates. This step proves to require the presence of aspartic acid, and differs from the previous step in that the nitrogen which is added to citrulline actually comes from the aspartic acid directly and not from metabolic ammonia. This reaction also requires an input of energy either from simultaneous oxidative reactions or from ATP, but the exact point at which this energy is applied is not known. For this step clear evidence has been obtained that an intermediate complex is formed, though it has not yet been characterized completely. According to the present formulation the synthesis of arginine involves two reactions each having a specific enzyme. The first step gives rise to the intermediate complex which is believed to form as a result of the condensation of the enol form of citrulline with aspartic acid. In the subsequent reaction the intermediate is split to give malic acid and arginine. Since malic acid on oxidation forms the keto acid related to aspartic acid it would easily regenerate aspartic acid in tissues by oxidation followed by transamination.



With the information now available the urea cycle may be formulated somewhat more definitely as indicated in Figure 11.3.

¹³ Dr. Sarah Ratner (1903-) is Assistant Professor of Pharmacology of the New York University Medical College. Her chief research interest has been in the field of amino acid metabolism, and especially of late in the reactions of the urea cycle.

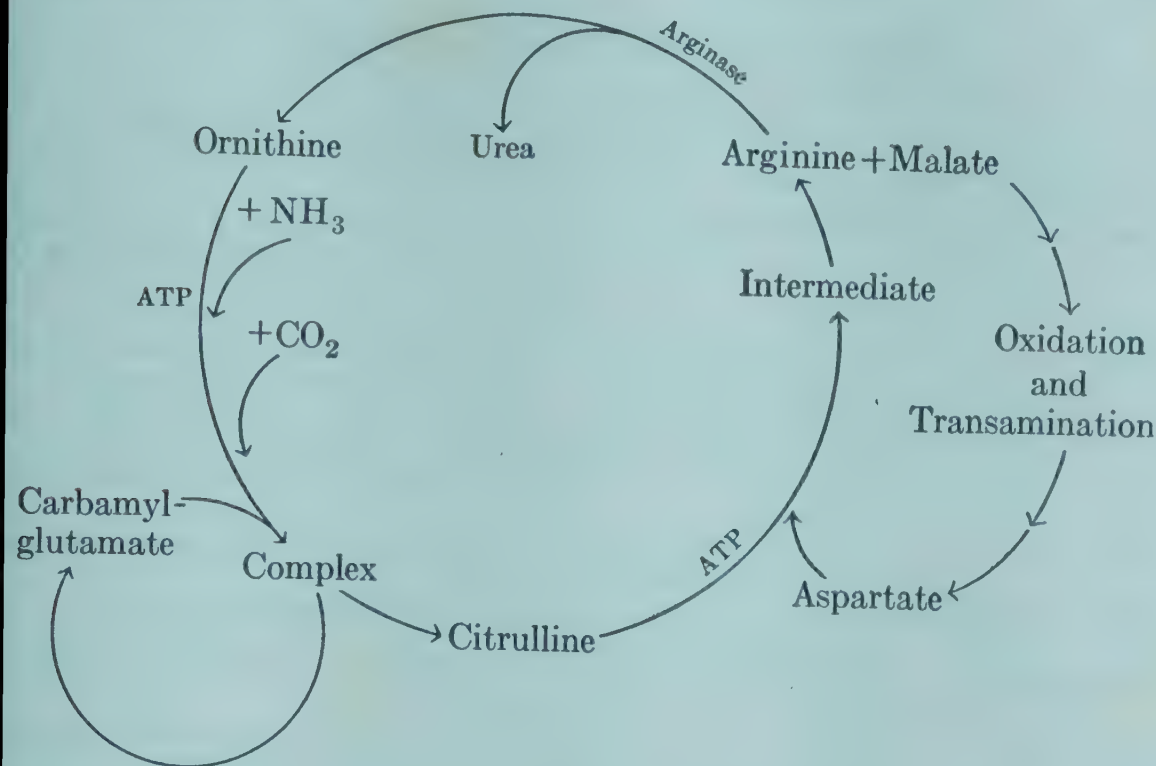


Figure 11.3. The urea cycle as modified by recent work on the individual reactions.

Metabolism of the Purines and Pyrimidines

The purine and pyrimidine bases are heterocyclic compounds related respectively to two "parent" substances which do not themselves occur naturally (see Chapter 5).

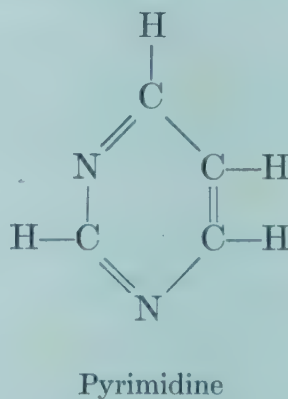
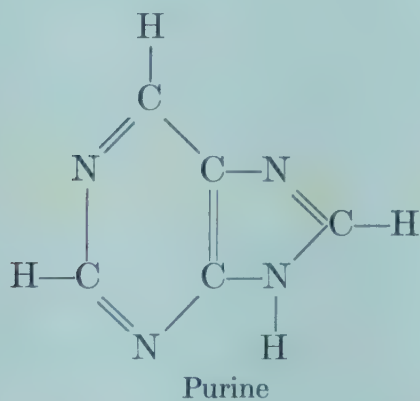


Table 11-VIII lists the important naturally occurring purines and pyrimidines.

OCCURRENCE OF THE PURINE BASES

The purine bases adenine and guanine occur in all living cells as constituents of the nucleoproteins. They are also found combined with ribose and phosphoric acid in the mononucleotides adenylic acid and guanylic acid. The purine in the other common mononucleotide, inosinic acid, is hypoxanthine. The structures of these compounds are given in Chapter 5.

TABLE 11-VIII. PURINE AND PYRIMIDINE BASES

Chemical Name ^a		Occurrence
PURINES		
Adenine	6-Aminopurine	In all cells in nucleic acids, and in nucleotides; free in plants; in ATP and in oxidative coenzymes
Guanine	2-Aminohypoxanthine	In nucleic acids and nucleotides; free in plants
Xanthine	2-Amino-6-oxypurine 2,6(1,3)-Purinedione ^b	Free in small amounts in plants and animals
Hypoxanthine	2,6-Dioxypurine 6(1)-Purinone	In inosinic acid in muscle; free in small amounts
Uric acid	6-Oxypurine 2,6,8(1,3,9)-Purinetrioxone	In animal excreta and in plants
Heteroxanthine	2,6,8-Trioxypurine 7-Methylxanthine	In sugar beet
Theophylline	2,6-Dioxy-7-methylpurine 1,3-Dimethylxanthine	In tea leaves
Theobromine	1,3-Dimethyl-2,6-dioxypurine 3,7-Dimethylxanthine	In cocoa, cola, and tea leaves, and cocoa berries
Caffeine	3,7-Dimethyl-2,6-dioxypurine 1,3,7-Trimethylxanthine 1,3,7-Trimethyl-2,6-dioxypurine	In cocoa, cola, and tea leaves, and in maté
PYRIMIDINES		
Uracil	2,4(1,3)-Pyrimidinedione 2,4-Dioxypyrimidine	In plant and animal pentose (ribose) nucleic acids (PNA)
Cytosine	4-Amino-2(1)-pyrimidone	In plant and animal PNA
5-Methylcytosine		In nucleic acid of tubercle bacillus
Thymine	5-Methyluracil	In desoxyribose nucleic acid (DNA)

^a The first name in the list for each compound is the modern one, the ones which follow are in accordance with the earlier system of nomenclature.

^b The number in parentheses indicates the nitrogen of the ring to which the added hydrogen is attached when a neighboring carbon becomes part of a keto group.

The unique importance of adenine derives from its presence in several dinucleotides which function as oxidative coenzymes in all living cells. The substance we have designated FAD is one of these coenzymes, in which adenine mononucleotide is linked to a similar flavin compound; others will be discussed later in connection with biological oxidations. As far as is known adenine is the only purine which forms compounds showing this particular type of activity. Another adenine compound which holds a pivotal position in metabolism is the adenosine ¹⁴ triphosphate to which several references have already been made. In this compound the two last of the three phosphate groups are held in bonds of the high energy type, the energy for which has been derived from some previous oxidative

¹⁴ The names given the compounds in which a base is linked to a pentose molecule all end in -osine. Thus adenosine = adenine-ribose; inosine = hypoxanthine-ribose; etc.

process. When later these bonds are ruptured the energy they contain is set free and can be used for various endergonic reactions. ATP has been found in animal cells and in microorganisms but so far has not been definitely identified in plant tissues. A compound which appears to be a polymerized ATP has been found, however, in mung bean, and it seems likely that ATP or something very like it will prove to act in plants as a reservoir of energy as does ATP in a wide variety of other living cells.

The methylated purines are all xanthine derivatives and are confined to the plant world. No function for these compounds has been found; since their concentration in excised leaves does not fall with time they are clearly not used as a reserve food supply.

BIOSYNTHESIS OF THE PURINES

It was long supposed that the purine rings are synthesized in the body from urea to which its structure seems so clearly related. Isotopic experi-

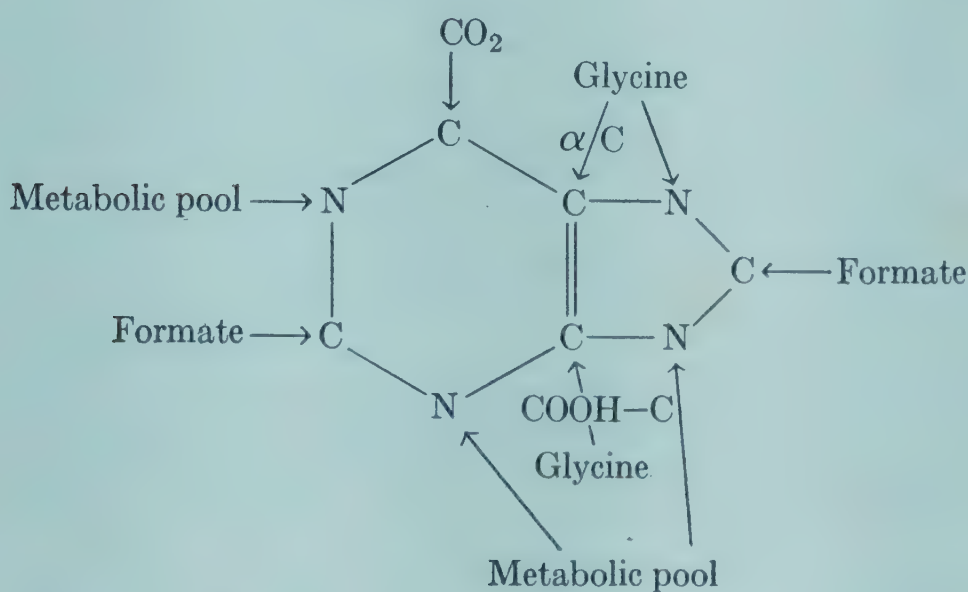


Figure 11.4. The precursors of the purine rings.

ments however have shown that injected labeled urea is excreted as such and does not lead to formation of labeled purines. Rather this complex ring structure is built up in the body from small molecules including the "active formate" previously referred to. Thus it has been shown that carbons 2 and 8 can be furnished either by formate or by glycine or by serine. Since both these amino acids are known to be in equilibrium with formate *in vivo* it is probable that it is actually formate which enters the synthesis in all three cases. Experiments with labeled molecules have shown that glycine provides atoms 4, 5, and 7, that carbon 6 is derived from carbon dioxide, and that the three nitrogens other than that in position 7 are taken from the metabolic pool. While it is not possible at this time to give the sequence of steps by which living cells build up the purine

rings, the diagram in Figure 11.4 indicates the sources of the component atoms of the purine skeleton. Most of this work has been done either with the enzymes of animal tissues or with those of microorganisms, but these show so uniform a pattern that it seems likely that this same pattern will be found operative in the plant world also. Little is known about the synthesis in plants of the methylated derivatives of xanthine, but there is some evidence that they do represent a progressive methylation through the mono- and dimethyl derivatives to caffeine with its three methyl groups.

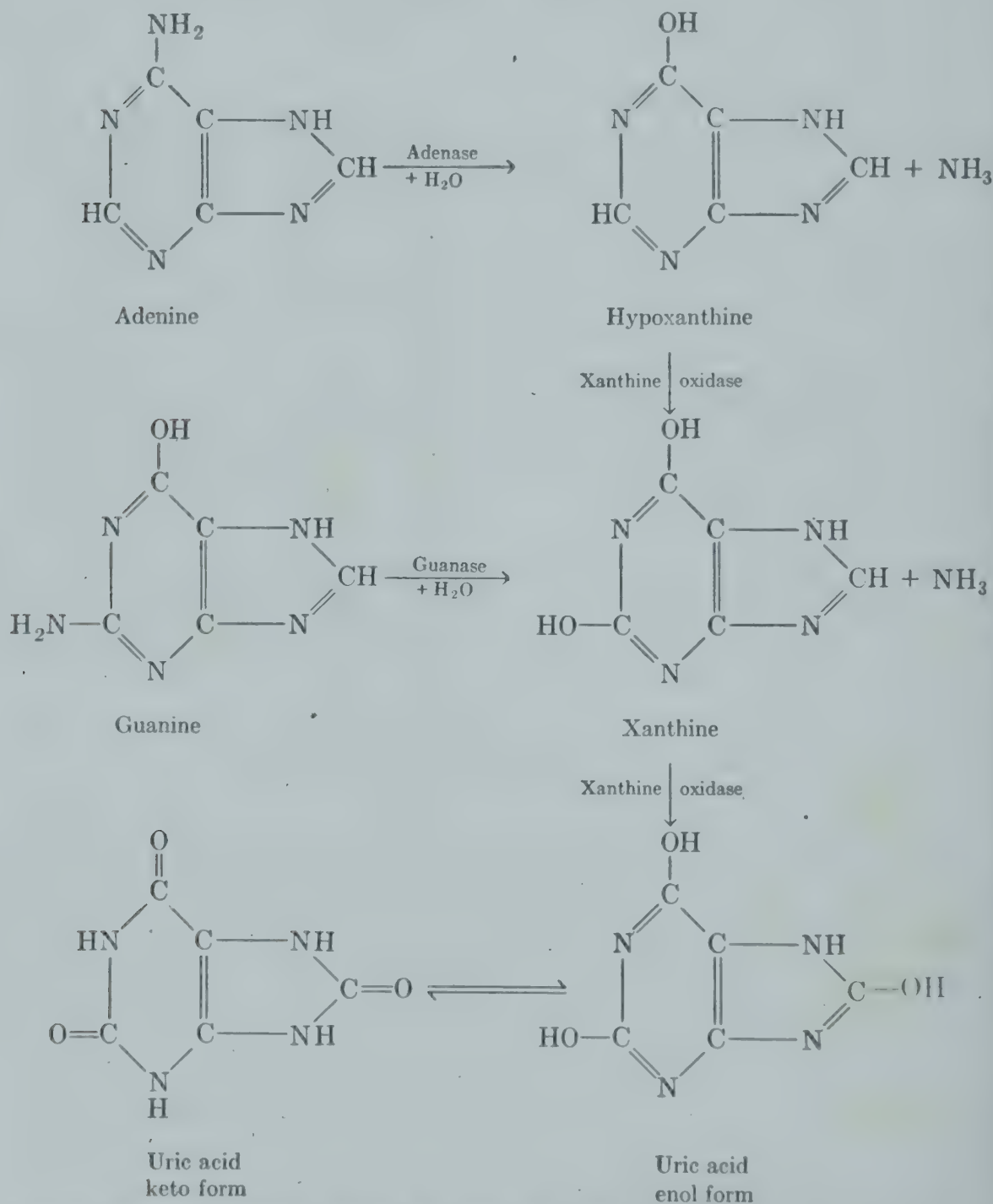


Figure 11.5. The metabolic reactions by which the purines are transformed into uric acid.

THE PYRIMIDINE BASES

The pyrimidine bases are found in small amounts in the nucleic acids. Uracil and cytosine occur in ribonucleic acid, usually abbreviated to PNA for "pentosenucleic acid." Thymine and cytosine are the pyrimidine components of desoxypentosenucleic acid (DNA).

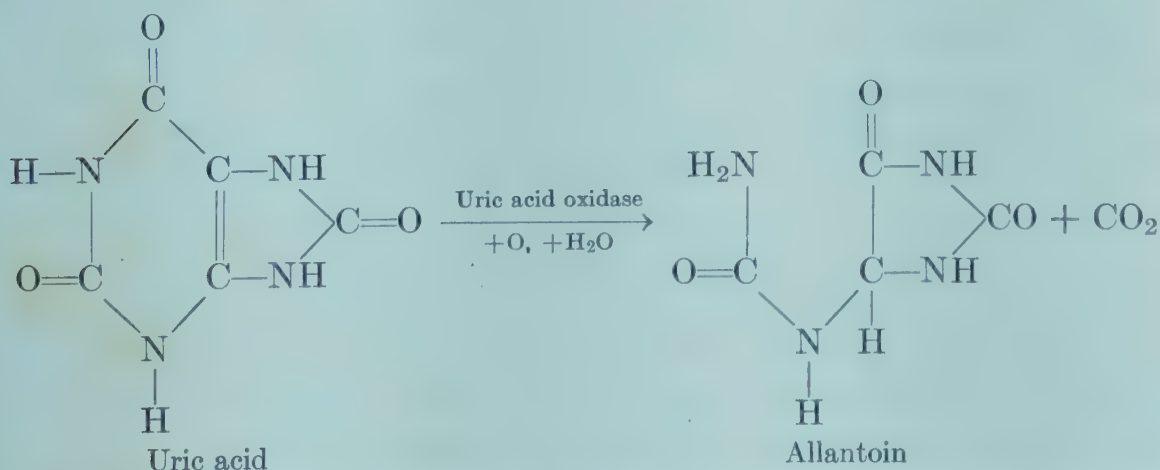
Almost nothing is known of the biosynthesis of the pyrimidines either in plants or animals, though there is some evidence that the compound is built up from small units and that the final ring closure is preceded by a linking of the pyrimidine precursor to ribose or other pentose.

PURINE EXCRETION

The purine skeleton appears in urine and other excreta of animals in the form of the trioxypurine, uric acid, or of closely related compounds.

Formation of Uric Acid and Allantoin by Animals. In man the chief nitrogenous excretory product is urea, with uric acid, which accounts for less than 1 per cent of the total nitrogen of the urine, representing the end product of purine metabolism. As the nucleoproteins, the coenzymes, and other purine compounds are replaced in the cell mechanism their constituent purines are split out and carried to the liver and there transformed into uric acid. The chart in Figure 11.5 indicates the paths by which the various deaminations and oxidations are carried out. It should be noted that these deaminations catalyzed by the enzymes adenase and guanase are simple hydrolytic reactions and not the oxidative deaminations characteristic of the amino acids.

Although in man and the higher apes, uric acid is excreted as such, in most other mammals uric acid is further degraded. Under the influence of the enzyme uricase, it is oxidized to the more soluble allantoin, which is then excreted.



To this latter statement there is however one strange exception. About 1915 Stanley Benedict¹⁵ discovered that the Dalmatian coach hound

¹⁵ Stanley R. Benedict (1884–1936) was Professor of Physiological Chemistry at Cornell University Medical College in New York City. His outstanding contributions

alone among dogs excretes uric acid instead of allantoin. Why the primates and this one breed of dog should thus share the misfortune of being subject to gout is a mystery. Being incapable of oxidizing the relatively insoluble uric acid, they may, if its excretion does not keep pace with its formation, deposit the excess in the joints. The tendency to form such deposits is apparently a familial matter, associated with an hereditary tendency toward high blood uric acid values. Normally the blood contains approximately 2–4 mg. of uric acid per 100 ml. In cases of gout the concentration may rise as high as 14 mg. Somewhat elevated values are often found also among close relatives of those who suffer from gout, who yet are themselves free of the disease. From this it is concluded that predisposition to these high values for blood uric acid is subject to hereditary transmission.

Uric Acid Synthesis in Birds and Reptiles. In birds and some reptiles uric acid is the major nitrogenous constituent of the excreta, which contain little or no urea. This has been explained as an adaptation of these forms to a relative water shortage. Thus it is assumed that excretion of ammonia is the simplest and most primitive arrangement for getting rid of waste nitrogen. This is perfectly feasible so long as an animal living in water can wash away ammonia with a copious stream of water. But ammonia is a toxic substance and life on dry land was hardly possible until some mechanism had been developed for transforming it into something less noxious. One group of organisms, including the primates, the amphibia, and the elasmobranch fish, solved the problem by converting the ammonia into urea which is comparatively innocuous and is excreted in the urine at such a rate that its concentration in the body fluids never becomes dangerous. But the birds and some of the reptiles had a double problem. Not only must their own bodies adapt to a certain scarcity of water, but their eggs are enclosed in shells or tough membranes which exclude water entirely. During the development of the embryo nitrogenous wastes form from the stored reserves of protein foods and if these were allowed to accumulate as ammonia or as urea their concentration inside the shell would soon become lethal. This unhappy result is avoided because these organisms transform metabolic ammonia into the harmless and insoluble uric acid which precipitates and is thus effectively removed from the fluids of the embryo. The assumption is that this mechanism, essential to survival inside the egg, is carried into adult life. Animals which excrete the major part of their waste nitrogen as uric acid are said to be *uricotelic*, while those which excrete predominantly urea are *ureotelic*.

The synthetic formation of uric acid, as opposed to the degradative one used by mammals, comes about as noted above by condensation of small

were in the field of analytical methods, adapted to the estimation of blood and urine constituents.

molecules, ammonia, formate, glycine, and carbon dioxide. In fact most of the work on which our present knowledge of purine synthesis is based has actually been concerned with uric acid synthesis as catalyzed by the enzymes of bird liver. Beyond the fact that this synthesis requires only the four small molecules just enumerated, most of our information about it is negative. It has been shown that urea is not an intermediate in uric acid synthesis, nor is xanthine or hypoxanthine. Whether this last means that synthesis of the purine skeleton follows entirely different pathways when other purines than uric acid are the objective is also not known. This and many other questions must be answered before the steps in this fundamental synthesis can be written down as a series of definite chemical reactions.

Analysis of the body proteins of pigeons after administration of isotopic ammonia, revealed the fact that the marker not only appeared in excreted uric acid but had entered the purines and pyrimidines of the nucleic acids which are part of the indispensable nucleoproteins in every living cell. This suggests that the nucleic acids of the body are labile in the same manner as are the proteins. Purine and pyrimidine bases seem to slip in and out of the nucleic acids as amino acids slip in and out of proteins. While free, the purines and pyrimidines acquire new nitrogen atoms from the metabolic pool, fitting them into the rings as well as using them to replace amino groups. The regenerated bases may then go back into the organ nucleic acids, and thence into the nucleoproteins, or they may be oxidized to uric acid and excreted.

Putrefaction and Detoxication

In the preceding paragraphs we have been concerned with the fate of nitrogen compounds after absorption. But although most of the protein in a meal reaches the circulation in the form of amino acids, it may happen that some small part of the digested food mass has moved into the lower intestine before it can be absorbed. There the bacterial population is high, and these bacteria elaborate active decarboxylases which act upon amino acids according to the following general equation.



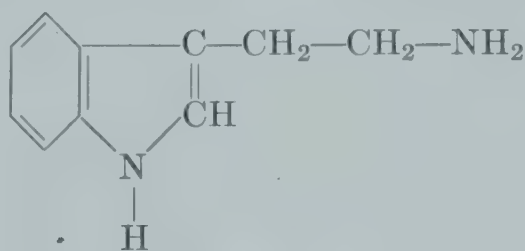
The amines which are formed are more or less toxic, and when they are absorbed may give rise to "autointoxication." The body meets this emergency by detoxicating and excreting the offending molecules, and so long as excretion keeps pace with absorption there are no ill effects. Six bacterial decarboxylases have been identified, each specific for a single natural amino acid.

Table 11-IX lists these acids and gives the formulas of the corresponding amines. Although the enzyme has not been isolated, it has been

TABLE 11-IX. AMINES FORMED BY BACTERIAL DECARBOXYLASES

Acid Acted Upon	Product of Decarboxylation
$\text{NH}_2-(\text{CH}_2)_4-\text{CH}(\text{NH}_2)-\text{COOH}$ Lysine	$\text{NH}_2-(\text{CH}_2)_4-\text{CH}_2-\text{NH}_2$ Cadaverine (Pentamethylenediamine)
$\text{NH}_2-(\text{CH}_2)_3-\text{CH}(\text{NH}_2)-\text{COOH}$ Ornithine	$\text{NH}_2-(\text{CH}_2)_3-\text{CH}_2-\text{NH}_2$ Putrescine (Tetramethylenediamine)
$\text{NH}_2-\underset{\text{NH}}{\underset{ }{\text{C}}}-\text{NH}-(\text{CH}_2)_3-\text{CH}(\text{NH}_2)-\text{COOH}$ Arginine	$\text{NH}_2-\underset{\text{NH}}{\underset{ }{\text{C}}}-\text{NH}-(\text{CH}_2)_3-\text{CH}_2-\text{NH}_2$ Agmatine
$\text{HO}-\langle \text{Cyclohexyl} \rangle-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$ Tyrosine	$\text{HO}-\langle \text{Cyclohexyl} \rangle-\text{CH}_2-\text{CH}_2-\text{NH}_2$ Tyramine
$\text{HC}=\underset{\text{N}}{\underset{ }{\text{C}}}-\underset{\text{N}}{\underset{ }{\text{C}}}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$ Histidine	$\text{HC}=\underset{\text{N}}{\underset{ }{\text{C}}}-\underset{\text{N}}{\underset{ }{\text{C}}}-\text{CH}_2-\text{CH}_2-\text{NH}_2$ Histamine
$\text{HOOC}-(\text{CH}_2)_2-\text{CH}(\text{NH}_2)-\text{COOH}$ Glutamic acid	$\text{HOOC}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}_2$ γ -Aminobutyric acid

reported that intestinal bacteria also decarboxylate tryptophan to free tryptamine.

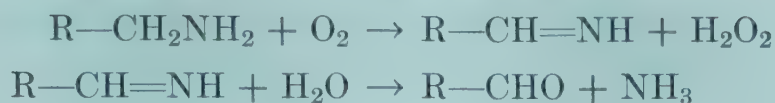


Tryptamine

DETOXICATION

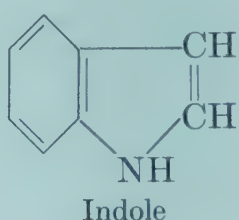
These toxic amines constitute one of a rather varied group of substances which must undergo some transformation before they are safe for transportation by the blood and excretion by the kidney. Some of the reactions by which this *detoxication* takes place have been mentioned in other connections, but they will be summarized here, since the proteins not only furnish some of the offending molecules, as in the instance just referred to, but also provide several of the compounds used for detoxication. In general a substance is detoxicated either by oxidation or by conjugation with another molecule which blocks its toxic grouping.

Oxidation. There are present in the cells of the liver a number of monoamine oxidases which act upon amines according to the following general equations, the first reaction being probably the only enzymic one:

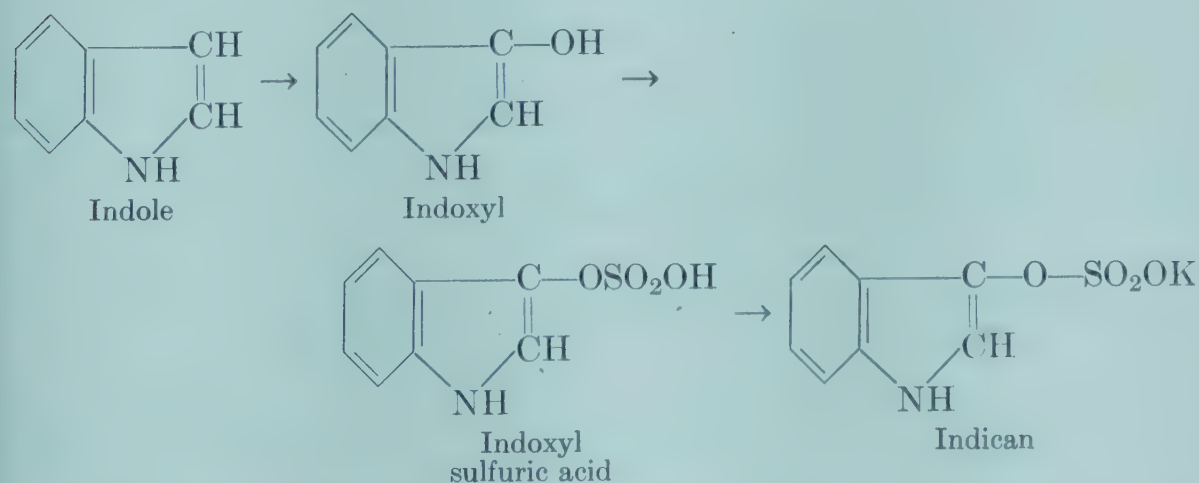


In this way the monoamines are transformed into nontoxic carbon compounds which can be further oxidized, and ammonia which is a normal metabolite. For the oxidation of the diamines, there is present in both kidney and liver a diamine oxidase which brings about the oxidation of a long list of diamines, including all those in Table 11-IX. The oxidations of some amines are complete, and lead to formation of carbon dioxide, water, and ammonia. With others the oxygen uptake is not great enough to indicate complete oxidation, though even here any ring structures seem to have been destroyed.

Conjugation with Acids. 1. *Sulfuric Acid*: Bacterial enzymes acting upon tryptophan or tryptamine produce two evil-smelling compounds, skatole and indole, said to be largely responsible for the odor of feces.



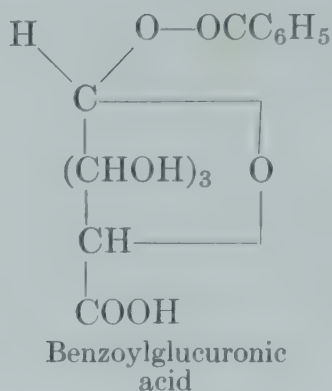
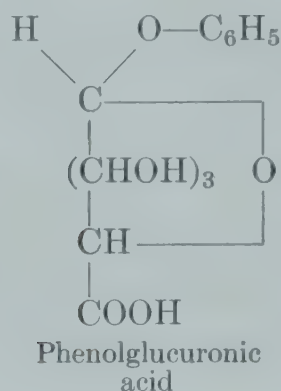
If these substances are absorbed, they are oxidized in the liver to hydroxyl compounds, which after conjugation with inorganic sulfate are excreted in the urine as potassium salts of the so-called "etheral sulfates." The steps in the transformation of indole are indicated below.



Phenolic hydroxyl groups may also be detoxicated by conjugation with sulfuric acid.

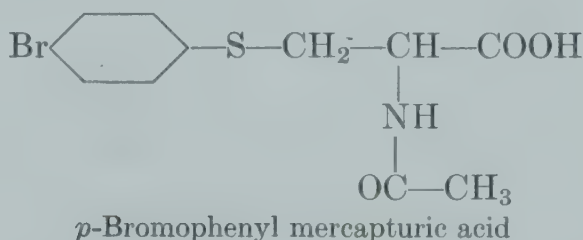
2. *Glucuronic Acid*: By a mechanism which has not been identified, the body synthesizes glucuronic acid, $\text{CHO}-(\text{CHOH})_4-\text{COOH}$, which is used

to detoxicate aromatic acids and phenols, forming with either type of compound a glucosidic link.



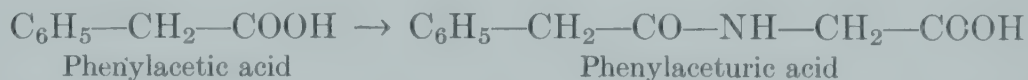
Thus phenolic compounds may occur in the urine either as ethereal sulfates or as glucuronates.

3. *Cysteine*: Certain aromatic compounds are excreted in the form of mercapturic acids after being conjugated with acetylated cysteine. For example, administration of bromobenzene to dogs is followed by excretion in the urine of *p*-bromophenyl mercapturic acid.



4. *Acetic Acid*: Toxic amino groups are sometimes acetylated before being excreted. For example, unnatural amino acids as well as aniline and sulfanilamide are excreted in the acetylated form.

5. *Glycine*: Detoxication of aromatic acids is frequently achieved by conjugation with glycine. Reference has already been made to the formation of hippuric acid from benzoic acid (see p. 161). Other aromatic acids form similar detoxication products.



The body is able to synthesize glycine rapidly enough to detoxicate even very large doses of aromatic acid, which appear in the urine within the following twenty-four hours.

As far as we know most of the work of detoxication is carried out by the liver. The proteins not only furnish glycine and cysteine as detoxicating agents, but are probably responsible for the sulfate as well. Oxidation of cysteine yields as one product inorganic sulfate, part of which is excreted

as such, but part of which can be used for conjugation when the necessity arises. It is a striking thing that the body is not only equipped to deal with such toxic material as it would normally have to meet when eating a normal diet, but seems to have mechanisms in reserve for dealing with such completely foreign substances as bromobenzene and sulfanilamide.

Suggestions for Further Reading

GENERAL

Nitrogen Metabolism of Animals

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Schoenheimer and his colleagues have a series of very interesting and informative papers in *J.B.C.*, 127, 130, and 131, 1939, reporting on the results of the early isotope experiments.

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ESSENTIAL AMINO ACIDS

A series of papers by W. C. Rose and his colleagues has reported on the amino acid requirements of rats, and is in process of defining the requirements of man. The papers cited below will give references to earlier work:

J.B.C., 182:541, 1950;

188:49, 1951;

193:605 and 613, 1951;

196:33, 1952.

ROSE, W. C., "The Nutritive Significance of the Amino Acids," *Physiol. Revs.*, 18:109, 1938.

Study Questions

1. In Schoenheimer's experiment with the feeding of labeled amino acids, what startling facts emerged?
2. Why was the experiment in which labeled tyrosine was added to an already adequate diet particularly interesting?
3. State briefly the metabolic pattern which is indicated by the experiments of Schoenheimer.
4. What enzymes are known to catalyze the oxidative deamination of amino acids? Write equations for the reactions by which this is brought about, indicating which reactions are enzymic and any that are not. For which amino acids are there specific oxidases?
5. What does FAD stand for and how does it act?
6. What is believed to be one function of asparagine and glutamine? Write the formulas for these compounds.
7. What is the transamination reaction? Who discovered it? What is the distribution of transaminase? What is its coenzyme? Which part of the Schoenheimer results is clarified by knowledge of this reaction?
8. Which substances are known to take part in transmethylation? What is the importance of this reaction?

9. Describe the series of experiments in which the biosynthesis of creatine was elucidated. What evidence was there that such a synthesis must be taking place in animals?

10. What are "ketogenic" amino acids, and how were they identified?

11. What is meant by the "metabolic pool of amino acids"?

12. What was the experimental evidence which led Krebs to postulate a "urea cycle"? Sketch this cycle in the simple form in which Krebs first proposed it, including the formulas of the three amino acids involved.

13. What substances have been found to contribute to the biosynthesis of the purines?

14. Write the formulas for: adenine, guanine, ATP.

15. List the compounds which are used in the animal body for detoxicating various substances and indicate the type of compound with which each reacts.

Lipid Metabolism

I think it is worth your while to consider whether a new line of advance in biology . . . may not come from researches motivated by a full conviction that each observed event in the complex behaviour of a living system is associated with equally complex and, surely, equally biological, events at the molecular level of its constitution.

F. G. HOPKINS: Linacre Lecture (1938)

The fats are an essential part of the animal diet, in which they provide a supply of easily oxidizable substrates. Recent evidence indicates furthermore that even with a diet which makes other adequate provision for energy needs at least 16 per cent of the caloric intake must be in the form of fat if growth is to be normal.

Investigation of the metabolic changes undergone by the lipids has been beset with difficulties. The fats themselves are too insoluble in water to be used readily as experimental substrates. Yet the soluble soaps which they yield on hydrolysis are so toxic to living tissue that they can be used only in very low concentrations, and the calcium and magnesium soaps are so insoluble that the ionic ratios in a nutrient solution are upset by precipitation if fatty acids are added. At the same time many of the enzymes involved in lipid metabolism have proved to be insoluble and hence difficult to extract and purify. The result is that much of the experimental work has had to be done with whole animals or at least with the intact cells of tissue slices into which the insoluble substrates diffuse with difficulty. Because of these difficulties the chemical information available about the lipids is meager compared with what is known about the proteins and the carbohydrates.

The Lipid Equilibrium in Animals

LIPIDS IN THE ANIMAL BODY

Except as reserve food in the seeds, fats play a very small part in plant metabolism. In the animal on the other hand the fats constitute one of the chief reserve foods and the oxidation of fatty acids accounts for a substantial part of the oxygen uptake of many tissues. The body lipids are classified according to their location as either *structural lipids*, or *plasma lipids* or *depot lipids*.

Structural Lipids. It is a matter of common observation that the fat content of an animal can vary within wide limits, rising on a generous diet and falling rapidly during starvation. But even if the starvation is prolonged to the point of death the body lipids do not disappear completely. Apparently some fatty substances are so essential to the structure of living cells that they cannot be mobilized to serve as fuel even when the organism is in desperate need of oxidative substrates. The lipid material which is thus conserved is sometimes referred to as the "constant element" as opposed to the rest of the body lipids which are "variable." The constant, structural lipids consist very largely of compound lipids and sterols, with the relative proportions differing from one tissue to another. Thus for example nervous tissue is especially rich in phospholipids and contains also the major part of the cerebrosides of the body.

Plasma Lipids. Beginning about two hours after a meal, the blood plasma becomes more or less milky in appearance due to the presence of finely emulsified neutral fat in the form of chylomicrons. In the course of a few hours this alimentary *lipemia*, or excessive fat concentration in the blood, disappears. Besides this very variable concentration of neutral fat in process of transportation from the intestines, the plasma lipids include also cholesterol and phospholipids. The cholesterol is partly free and partly esterified with fatty acids; the phospholipids are largely phosphocholines. Table 12-I gives for several species the average values for the

TABLE 12-I. AVERAGE LIPID VALUES IN BLOOD PLASMA OF NORMAL FASTING ANIMALS ^a

	Guinea Pig	Albino Rat	Rabbit	Cat	Man
Total lipid	169	230	243	376	530
Neutral fat	73	85	105	108	142
Total fat acids	116	152	169	228	316
Total cholesterol	32	52	45	93	152
Ester cholesterol	21	31	23	63	106
Phospholipid	51	83	78	132	165

Values are given in mg./100 ml. of plasma.

^a Data from E. M. Boyd, "Species Variation in Normal Plasma Lipids Estimated by Oxidative Micro-methods," *J.B.C.*, 143:131, 1942.

lipids of fasting plasma. Blood for such estimations is drawn in the morning before any food is eaten.

Recent experiments with the fractionation of serum proteins indicate that the greater part of the nondietary plasma lipids occur in the form of lipoprotein complexes. One such compound, with a molecular weight of about 1,300,000, included about 30 per cent cholesterol, 29 per cent phospholipid, and 23 per cent polypeptide besides some neutral fat and stably bound water. The behavior of the substance in the ultracentrifuge suggests a spherical molecule and its solubility in water can be explained by as-

suming that the lipid parts of the complex are concentrated as far as possible in the center of the sphere with the more soluble polypeptides making up more than half of the exposed surface. It is estimated that about 5 per cent of the total plasma protein is in the form of lipoprotein, and that such compounds account for a major part of the lipid fraction of the plasma. It will be recalled that the cell walls are also believed to consist of lipoproteins.

Depot Fats. When dietary fat leaves the blood stream, most of it goes into the fat depots. Of these the most important are in the subcutaneous connective tissue, the intramuscular connective tissue and the mesentery. This fat, which is deposited inside the walls of so-called fat cells, makes up the chief reserve fuel of the body. As contrasted with carbohydrates and proteins the fats are particularly well adapted to serve as a source of energy. In the first place they contain a smaller proportion of oxygen than is present in the other two types of foodstuff and therefore provide, per gram, more combustible carbon and hydrogen. As a result the energy set free in burning a gram of fat is over twice that obtained when the same weight of either carbohydrate or protein is metabolized. Thus the caloric value of 1 g. of glucose is 3740 calories, that of 1 g. of animal protein is 4250 calories, but combustion of 1 g. of an average fat yields 9450 calories.

As a body fuel the fats have another advantage. Because of their high percentage of hydrogen they produce when oxidized a larger amount of water than do equal weights of the other foodstuffs and this is often of great value to terrestrial animals. Those which, like the camel, must live under conditions of water shortage are able to satisfy their minimum requirements by relying for long periods upon oxidation of body fats for their energy supply.

Lability of Body Fats. To a generation plagued by the curse of overweight, few things have seemed more stable and permanent than animal depot fats. It therefore came as a great surprise when the first of the isotope experiments carried out by Schoenheimer and his associates showed that body fats are part of a very mobile equilibrium in which they are continually being reconstituted from fats of the diet. For example, in their very first experiment a fat was labeled by partially saturating the double bonds in olive oil with deuterium. This oil was then fed to mice in such limited amounts that they actually lost weight. According to all the older theories of fat metabolism they should have oxidized all of the dietary fat as well as part of their lipid reserves. Yet when the animals were sacrificed after several days the greater part of the deuterium was still present, either in the depot or the organ fats. Later work has abundantly confirmed what was foreshadowed in this experiment: that the body fats are in a state of rapid flux, being continually broken down for fuel and reconstituted from the metabolic pool. The time required for the com-

plete renewal of the depot fats probably varies widely from one animal to another, but Rittenberg and Schoenheimer estimated that in mice half of the depot fats were reconstituted in the course of seven days, while more than half of the fatty acids present in the liver had been newly incorporated within one day. The rapidity of this process in liver tissue is but one of many indications that the liver plays a unique role in fat metabolism.

Some indications of the way in which this mobile lipid equilibrium functions have been obtained in experiments which showed (a) the readiness with which fatty acids undergo minor transformations, and (b) the speed with which body water is incorporated synthetically into fatty acids.

Palmitic acid labeled with deuterium stably bound to carbon was mixed with butter and fed to rats as part of an adequate mixed diet. After eight days it was found that 44 per cent of the deuterium administered was still present in the body fats. Of this more than half was present as palmitic acid, as might have been expected if food fatty acids are used directly in synthesis of body fats. But there were also present labeled stearic, myristic, lauric, and palmitoleic acids. The presence of stearic acid meant that the carbon chain of palmitic acid had been lengthened by two carbons to give an acid with eighteen carbons. Myristic and lauric acids resulted from removal of two and of four carbon atoms respectively, while the presence of unsaturated palmitoleic acid indicated that two hydrogens had been removed from palmitic acid. It should be noted that none of these reactions took place because the animal had need of these particular acids. The butter of the diet included adequate amounts of all of them. Evidently the transformation mechanism acts continuously and automatically, providing a supply of mixed fatty acids from which the body fats are regenerated.

Side by side with the utilization of dietary fatty acids, other dietary constituents contribute to the body lipids through a continuous synthetic process in which body water contributes hydrogen atoms for the reduction of the carbon chain. This was first proved by feeding or injecting heavy water which distributes itself rapidly through all the body fluids. Thus any chemical reactions in the cells take place in a medium which contains a certain amount of water labeled with deuterium. In the course of a few days the fats of the body were marked with the heavy isotope and analysis showed that it was present in stearic and palmitic acids as well as in some unsaturated acids. The labeled unsaturated acids did not include linoleic acid, and this was not surprising, for earlier experiments had indicated that linoleic acid with its double bonds at positions 9 and 12 is an "essential" fatty acid, and must be provided in the diet. This can only mean that the body is incapable of synthesizing this particular compound.

To summarize: the cells are continually degrading their stores of fat and simultaneously replacing these stores by synthesis, using both the fat acids

of the diet and other acids which have been newly formed. These latter may result from comparatively minor changes in preëxisting fat acids, by which the chain is lengthened or shortened, dehydrogenated or saturated at a double bond. New acids are also synthesized from the various nonfat precursors. The evidence indicates that these syntheses make use of some two-carbon fragment to build up the acid chains and that about half of the hydrogen of the finished product comes from body water. The fact that the incorporation of isotopic hydrogen is much faster in the liver than in other tissues is another fact pointing to the predominant role played by the liver in fat metabolism.

INTERCONVERSIONS OF THE FOODSTUFFS

In general, as was noted in Chapter 6, each species of animal on a normal mixed diet lays down a characteristic type of fat. Thus mutton fat is grossly distinguishable from pig fat and proves to contain certain definite fatty acids in an approximately constant ratio. On the other hand it is possible, by feeding large amounts of a given fat, to modify very materially the characteristic pattern of a particular species. In the data in Table 12-II both tendencies are illustrated. The animals are clearly changing

TABLE 12-II. INFLUENCE OF FOOD FAT UPON BODY FAT OF RATS ^a

Food Fat (60% of total calories = fat; 40% of total calories = skimmed milk)	Iodine No. of Food Fat	Iodine No. of Body Fat (Average value for 6 animals)
Soybean oil	132.3	122.5
Corn oil	124.3	114.2
Cottonseed oil	108.1	107.4
Crisco	78.8	81.8
Butter fat	35.8	55.5
Cocoanut oil	7.7	35.3

^a Data from Anderson, W. E., and Mendel, L. B., "The Relation of Diet to the Quality of Fat Produced in the Animal Body," *J.B.C.*, 76:729, 1928.

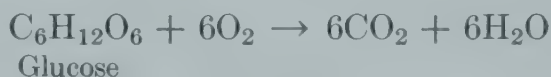
somewhat the pattern set by the food fats, since the iodine numbers of the body fats are neither as high nor as low as those of the foods which have extreme values. On the other hand the unsaturation of the body fats does reflect the degree of unsaturation of the foods, as if some at least of the fatty acids of the diet had been incorporated unchanged in the body fats. Later work with isotopic tracers has shown that the dietary acids which contain ten carbons or less are normally oxidized completely but that some of the longer acids are used directly in synthesis.

Although a high fat diet may thus lead to an almost exclusive use of preformed acids in fat synthesis, on a mixed diet only a part of the body

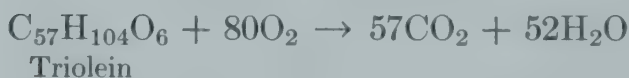
fats arise directly from the fats of the food. Normally, both carbohydrates and proteins contribute to the synthesis of fat acids.

Carbohydrate as Lipid Precursor. It is a commonplace observation that carbohydrate in the diet leads to deposition of depot fats. And indeed as long ago as 1860 there was carried out with pigs one of the first biochemical balance experiments, designed to test this very point. By comparison of the amounts of fat, protein, and carbohydrate in the diet with the amounts of each which were either oxidized, excreted, or stored in the carcasses, it was proved conclusively that carbohydrate serves as a precursor of body fat. Since then isotope experiments have shown not only that animals and microorganisms synthesize labeled fats from labeled glucose, but that in the normal metabolism of glucose this transformation is of quantitative importance.

The *respiratory quotient* or "R.Q." of living tissue is often taken as an index of the extent to which certain foodstuffs are being oxidized. The respiratory quotient is defined as the ratio of the volume of carbon dioxide evolved by an animal or tissue to the volume of oxygen consumed. In order to obtain a theoretical figure for pure carbohydrates, proteins, and fats it is necessary to assume an average formula for the two latter. In the following equations this has been done, using glucose as a representative carbohydrate and triolein as a typical fat.



$$\text{R.Q.} = \frac{6\text{CO}_2}{6\text{O}_2} = 1$$



$$\text{R.Q.} = \frac{57}{80} = 0.71$$

In order to obtain a comparable figure for proteins, allowance must be made for the fact that some of the molecule is eliminated as urea, leaving only a part of the carbon to be oxidized to carbon dioxide and water. Figures for an average protein indicate that 1 g. of urinary nitrogen corresponds to the metabolism of 6.25 g. of protein, the absorption of 5.91 l. of oxygen and the evolution of 4.76 l. of carbon dioxide:

$$\text{R.Q. for protein} = \frac{4.76}{5.91} = 0.801$$

Thus an R.Q. of 1 indicates the exclusive combustion of carbohydrate and one as low as 0.7 shows that the oxidative substrate is fat. From intermediate values it is possible, if the nitrogen excretion is known, to calculate

the relative amounts of each of the primary foodstuffs which are being metabolized.

If there is an extensive conversion of carbohydrate to fat the respiratory quotient also reflects this. Formation of fatty acids from sugars is a reductive process, leading to an R.Q. higher than 1. Assuming that glucose is to be converted to stearic acid, one possible equation is the following:



In such a reaction the respiratory quotient is $12/4$ or 3. Other equations can be written in which larger relative amounts of carbon are oxidized to carbon dioxide, as for example



Here the R.Q. is 1.36. Whatever representative equation is chosen the figure is greater than 1. Experimental results with living organisms which are known to be converting sugars to fat show that this process is accompanied by elevated values for the respiratory quotient. Rats on a diet composed exclusively of dextrin have R.Q. values of 1.5 to 2.0 while yeasts which are using sugars as substrates for fat synthesis give R.Q. values of 1.15 to 1.50.

The mechanism of the synthesis of fatty acids from carbohydrates can best be considered later when it will be possible to see it in its relation to other biosynthetic processes. Among the facts which must be taken into account in any attempt to formulate the course of the reaction the following should be noted.

1. Biological synthesis of fatty acids from carbohydrates is an aerobic process, indicating that it can only take place if there is a simultaneous exergonic reaction going on.
2. The presence of phosphate appears to be essential, though it is not yet clear whether this substance is needed for the breakdown of the carbohydrates or for the synthesis of the fats.
3. Saturated fatty acids seem to be the first products of fat synthesis. This is why livestock producers feed their animals a high carbohydrate diet to "harden" the body fats just before sending them to market.
4. Fat synthesis is favored by the presence of some of the B vitamins. Thiamine is essential to the process in animals and in some microorganisms, and others of this group of vitamins seem to augment lipid synthesis.

Fats from Proteins. Fat formation from carbohydrate is quantitatively significant and is furthermore a matter of commercial importance since it makes it possible for farmers to feed animals the cheapest type of food

and to recover the relatively valuable fat. But the conversion of protein to fat is an expensive procedure and therefore economically wasteful. That it does take place has been proved by feeding previously starved animals on a diet which consisted almost exclusively of protein. After about four weeks the animals had regained their normal weight and of the total increase in weight about 36 per cent was fat. Clearly then when conditions require it in order to provide a minimum of fat for metabolic purposes, the animal organism is able to use the amino acids as a carbon source and to synthesize fatty acids from them.

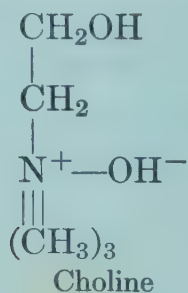
Conversion of Fatty Acids to Carbohydrates. For many years there was no proof that animal cells could reverse the usual transformation and bring about the synthesis of carbohydrates from fats. Studies of the respiratory quotients of hibernating animals indicated that this process was a likely one in animals using their fat reserves as a sole source of nourishment, but there was no direct experimental proof that this was so. It is therefore of some interest to note that it has very recently been reported that when fatty acids labeled with C^{14} are administered to rats the radioactive carbon is incorporated both into glucose and into glycogen.

ROLE OF THE LIVER IN LIPID METABOLISM

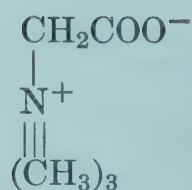
About 5 per cent of the total dry weight of the normal liver consists of lipid material, chiefly glycerides and phospholipids. But in various clinical disorders including diabetes and acute yellow atrophy of the liver the amount of fat may reach 50 per cent of the total weight, changing completely the appearance and texture of the organ. Such *fatty livers* can be induced experimentally by starvation, by administration of liver poisons such as carbon tetrachloride or phosphorus, or by excessive feeding of fat or cholesterol. Apparently all these diverse conditions share the property of disrupting normal fat metabolism. In the course of the search for the cause and for means of preventing fatty livers much has been learned of the ways in which the lipids are normally metabolized.

Fatty Liver and Lipotropic Factors. The fatty infiltration of the liver which accompanies the diabetes in depancreatized animals is much reduced if the animal is fed raw pancreas. This was at first attributed to the replacement of the pancreatic digestive enzymes and the resulting improvement in fat digestion. But Best¹ and his colleagues showed that the same curative effect was exerted by the mixed phospholipid fraction of pancreatic tissue, and later that either lecithin or choline or the closely related betaine could replace this tissue extract. A substance which, like choline, cures or prevents fatty liver under conditions in which it tends to appear is said to exert a *lipotropic* action.

¹ See page 437.



Choline



Betaine

A second compound which proved to be a lipotropic agent is casein. Fractionation of this protein and of others which had a curative effect indicated that the extent of the lipotropic action was proportional to their methionine content. It will be recalled that methionine is one of the indispensable amino acids and that it takes part in the biosynthesis of choline by providing labile methyl groups. Thus its action as well as that of betaine in the prevention of fatty liver probably depends upon their activity in promoting synthesis of choline, which in turn is needed for formation of phosphocholine (lecithin).

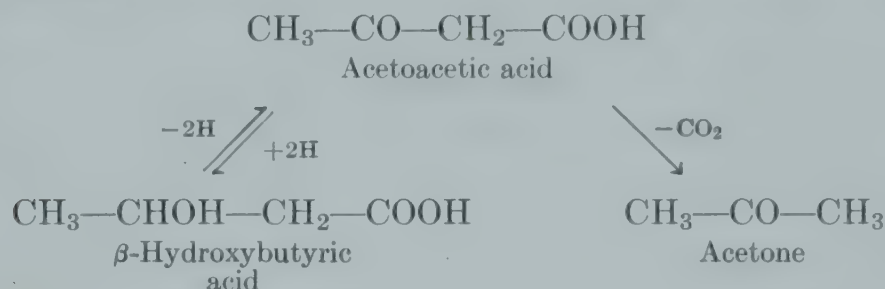
Still another lipotropic agent is inositol. Since the phospholipid fraction which was originally called "cephalin" is now known to include more than one phosphoinositol, the efficacy of inositol itself can be explained in terms of its promotion of phospholipid synthesis. Indeed there is some experimental evidence that ingestion of any of the lipotropic agents does increase the rate of synthesis of phospholipids, especially in the liver. Experiments which measure the rate of synthesis of a compound are said to measure its *turnover rate*, and are usually carried out by administering an isotopic tracer and determining the rapidity with which it is incorporated into some tissue constituent. When radioactive phosphorus is thus administered as orthophosphate it is found that its appearance in the liver phospholipids is more rapid in the presence of added choline than without it. But why the synthesis of phosphocholine should promote normal degradation of fat and so prevent its deposition in the liver in abnormal amounts is still to be discovered.

Several of the B vitamins have been shown to exert effects, adverse or favorable, on fat deposition in the liver, and several different hormones have also been connected with fat metabolism. But at the present moment there is no general agreement either on which hormones are involved, or on their specific functions. It seems clear that a normally functioning fat metabolism is the result of a complex balance of forces, many of which are still undefined. It is probable that the abnormality known as fatty liver may then arise as a result of any one of several different imbalances, some of which are rectified by administration of choline or inositol.

It is generally agreed that the liver is the focal point of fat metabolism and that the first step in the mobilization of depot fat for use is normally its transfer to the liver. When this transfer takes place under such con-

ditions that the fats cannot be metabolized as fast as they accumulate, the excess is deposited in the liver cells. The steps by which fats are normally degraded are just beginning to be understood and will be outlined in later sections of this chapter. The activity of choline and inositol in promoting normal metabolism suggests that a certain amount of phospholipid synthesis is an essential part of the process.

Ketogenesis. Another abnormal condition which is associated with the liver and with a deranged fat metabolism is the one known as *ketosis*, in which the three so-called "ketone bodies" appear in excessive amounts in blood and urine. These substances are acetoacetic acid, β -hydroxybutyric acid and acetone.



At the $p\text{H}$ of body fluids the acids are of course present chiefly as anions. Of these three, acetoacetic acid is believed to be the direct metabolite, giving rise to β -hydroxybutyric acid by a reduction reaction which is reversible and to acetone by an irreversible decarboxylation.

Early experiments indicated that the liver is the site of formation of ketone bodies and that they arise chiefly from fat acids. For example, various organs were perfused, but only the liver produced significant amounts of ketone bodies. Various possible precursors were tested, not only in perfusion experiments but in experiments with tissue slices and with whole animals, and it was only in the presence of fats or fat acids that detectable amounts of ketone bodies formed. This was hard to understand at first, since clinical ketosis generally occurs in conjunction with a disturbance of carbohydrate metabolism and can usually be alleviated by treatment which promotes the utilization of carbohydrate. Thus ketosis is a common accompaniment of severe diabetes and is alleviated by insulin therapy. These observations led at one time to the theory that fat acids can only be used if carbohydrates are being metabolized at the same time. Fats were said to "burn in the flame of carbohydrates." Medical textbooks listed different foods as "ketogenic" and "antiketogenic" and prescribed definite ratios of the two. The fats were considered ketogenic and the carbohydrates antiketogenic, but the position of a protein depended upon its constituent amino acids. Those amino acids which give rise to glucose were considered antiketogenic because of this. Other amino acids had, as we have seen, a definitely ketogenic effect and themselves gave rise to acetoacetate.

As a practical matter a proper control of the diet often lessened the formation of ketone bodies, but modern theory does not ascribe this to any subtle relation between fat and carbohydrate utilization. Nor is it now believed that acetoacetate is an abnormal product. The liver, and to a lesser extent the kidney, form acetoacetate whenever they oxidize fat acids. Normally this compound is carried in the blood to the muscles and to other organs where it furnishes a readily oxidizable substrate. It is only when the liver cells are for some reason having to depend almost exclusively on fats for energy that acetoacetate is poured out faster than the peripheral tissues can oxidize it. Part of it is then transformed into β -hydroxybutyric acid and acetone which appear in the urine and, in severe cases, impart to the breath the sweetish odor of acetone. In this connection it is interesting to note that the Eskimos have apparently adapted themselves successfully to a diet so high in fat that it would be very difficult for most people to tolerate.

The relations between normal fat metabolism, ketone body formation, and fatty infiltration of the liver may be summarized as follows:

1. Metabolism of the depot fats begins with their transport to the liver.
2. Some of the fat acids are there degraded in such a way that acetoacetic acid is one product. This substance is distributed in the blood to all the peripheral organs where it is oxidized or perhaps used in other ways.
3. When for any reason the ability of the liver to metabolize carbohydrates is lost or lessened, the resulting rapid mobilization of depot fat to supply energy, results in its being deposited in the liver faster than it can be used and the liver tissue becomes infiltrated with excess fat.
4. When the liver is forming acetoacetate faster than the peripheral tissues can use it, it is partially transformed into the other two ketone bodies, acetone and β -hydroxybutyric acid, and the three compounds then appear in blood and urine.

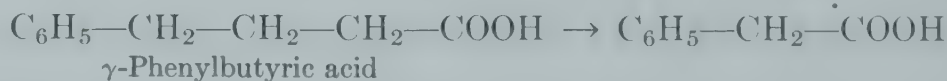
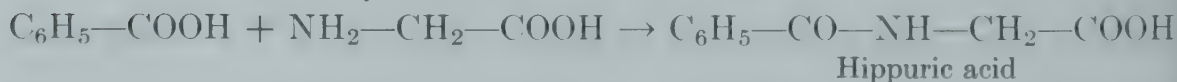
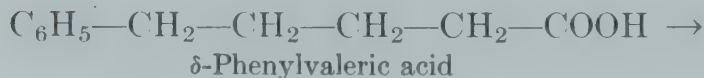
Chemistry of Lipid Metabolism in Animals

The first step in the catabolism of lipids is assumed to be hydrolysis, since most cells are supplied with lipolytic enzymes. In this process the simple fats yield glycerol and fat acids, while the conjugated lipids yield these same substances as well as phosphoric acid, nitrogenous bases and, in some cases, monosaccharides. Of these products orthophosphate is a constituent of all body fluids while glycerol and the monosaccharides will be considered in connection with carbohydrate metabolism. Almost nothing is known of the metabolic fate of the bases and so the study of the chemistry of lipid metabolism resolves itself into a study of the transformations undergone by the fat acids. These compounds are used as oxidative substrates not only by the liver but by the peripheral tissues as well, and are normally degraded to carbon dioxide and water. The major

problem has therefore been to determine the steps by which the long carbon chains are broken apart.

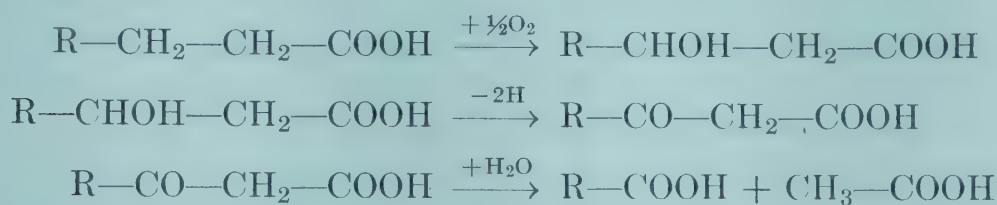
BETA OXIDATION

The first comprehensive theory of fatty acid oxidation was elaborated by Knoop about 1905, and was based upon the following experimental evidence. Long before the days of isotopic labels Knoop conceived the idea of marking a fatty acid with a phenyl ring which is not oxidized in the body but is excreted after conjugation with glycine. He synthesized a series of ω -phenyl fatty acids having 2, 3, 4, or 5 carbons in the side chain and fed these compounds to dogs. The aromatic compounds which he later isolated from the urine proved to be either one of two substances, depending on the length of the side chain in the acid administered. When the side chain had contained an odd number of carbon atoms, the nonoxidizable residue was excreted as hippuric acid, $\text{C}_6\text{H}_5\text{—CO—NH—CH}_2\text{—COOH}$. From acids with an even number of carbon atoms in the side chain phenylaceturic acid, $\text{C}_6\text{H}_5\text{—CH}_2\text{—CO—NH—CH}_2\text{—COOH}$, was synthesized and was recovered in the urine. From these results Knoop reasoned that the fatty acids were degraded by loss of two carbon atoms at a time and that when two more could not be removed without rupture of the phenyl ring the residue was conjugated with glycine and excreted. This process is known as β -oxidation since it is the β -carbon of one acid which becomes the carboxyl carbon of the next. The degradation is illustrated for an acid of each type in the following equations.



Because of the known relation of the ketone bodies to the fatty acids Knoop suggested that the intermediate steps in the removal of a pair of carbons from an acid involved formation of a keto acid. This explained the presence of acetoacetic acid as a metabolic product and was in accord

with the fact that fatty acids with uneven numbers of carbon atoms were known to give rise to fewer ketone bodies than did the even-numbered acids. The steps in the removal of a pair of carbons would thus involve in succession an addition of oxygen, removal of two hydrogens, and a final hydrolysis.

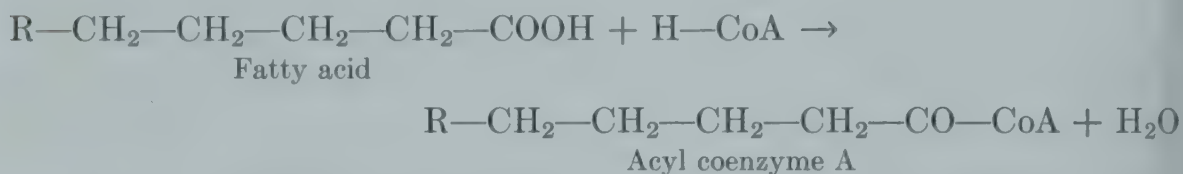


Whether or not exactly these events take place in this order, all subsequent work has confirmed the basic idea that fatty acids are metabolized by removal of two carbon atoms at a time. It will be recalled for example that when palmitic acid labeled with deuterium is metabolized in living rats, changes in chain length are always in units of two carbons which are added to or removed from the original acid. The two-carbon fragments which are split off were originally referred to as "acetic acid" but have recently been proved to be an acetyl derivative of the coenzyme A to which reference was made in connection with amino acid metabolism (see p. 366). Since all the naturally occurring acids have even numbers of carbon atoms the repetition of the series of reactions postulated by Knoop would eventually give rise in the penultimate step to one molecule of acetoacetic acid from each molecule of fat acid. If for any reason this compound failed to split into two two-carbon molecules its accumulation in the tissues might lead to formation of the two derivatives, β -hydroxybutyric acid and acetone and thus explain the development of ketosis. Isotopic evidence for this relationship between fatty acids and ketone body formation has been obtained with liver slices which form labeled acetoacetate from various acids marked with C^{14} . Furthermore, perfusion experiments have shown that it is only the acids with even numbers of carbon atoms which give rise to appreciable amounts of acetoacetate in liver tissue.

It was noted that Knoop's suggested mechanism would lead to formation of just one molecule of acetoacetate from each molecule of fatty acid, regardless of its chain length. When it became possible to label acids with isotopes this relationship could be investigated experimentally, and the results have led to a radical revision of the reaction mechanisms suggested by Knoop. In the first place it was found that in the living animal stanoic acid gives rise to more ketone bodies than an equimolar amount of butyric acid, which it should not do if each acid molecule forms one acetoacetate. Furthermore, with liver slices the amount of acetoacetate formed from fatty acids was definitely more than could be accounted for

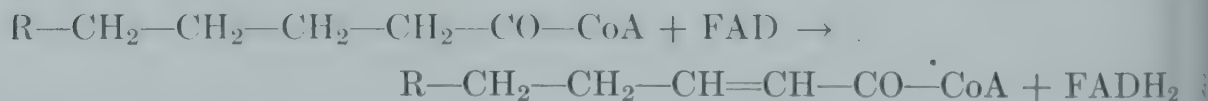
on this assumption and the odd-numbered fatty acids were found to give rise to small but significant amounts of ketone bodies.

In order to explain how acetoacetate can arise from a fatty acid in more than equimolar amounts and how an odd-numbered acid can yield acetoacetate by a process of β -oxidation, various modifications of Knoop's theory have been put forward. The one which is now generally accepted is known as the β -oxidation-condensation theory. According to this theory, oxidation of a fatty acid proceeds as Knoop suggested by successive removal of two-carbon fragments which are set free as acetyl derivatives of coenzyme A. The mechanism of this degradation has recently been elucidated by D. E. Green and his group. They have shown that it begins with the formation of an acyl coenzyme A complex by interaction of an intact fatty acid with the coenzyme. Allowing H-CoA to stand for coenzyme A, this reaction may be formulated:

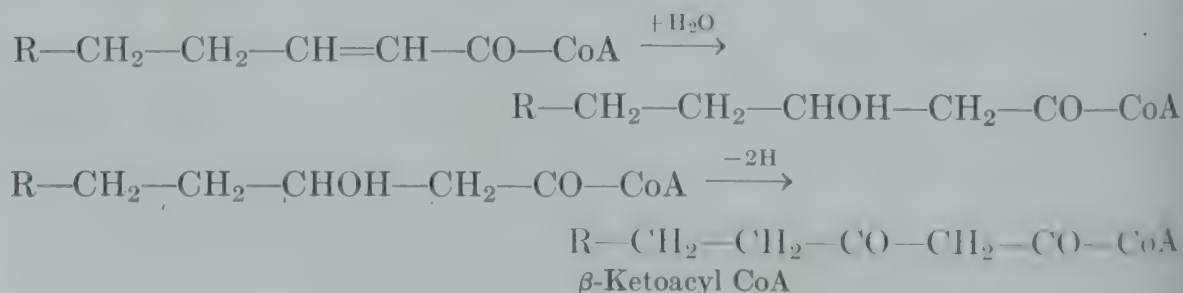


This step requires the presence of ATP which is split simultaneously to the monophosphate and free pyrophosphate, and is catalyzed by a so-called *activating enzyme*. Three enzymes of this sort have been isolated and prove capable between them of initiating the breakdown of all the natural fatty acids from the four-carbon one to the one with eighteen carbons.

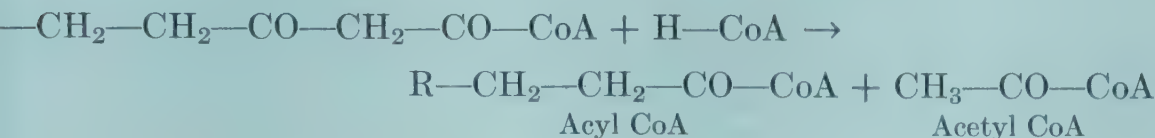
The second step is complex, but eventually yields a β -ketoacyl coenzyme A. It begins with desaturation of the acid chain, and for this reaction the obligatory coenzyme proves to be the same flavinadenine dinucleotide (FAD) which accepts two hydrogens from the D-amino acids. The unsaturated compound is then transformed into a β -keto derivative, pre-



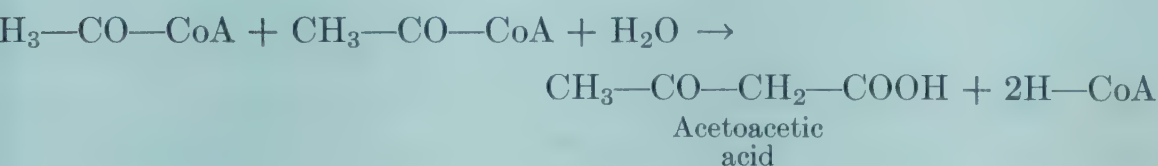
sumably through the addition of water to the double linkage, followed by dehydrogenation.



The keto derivative next reacts with a second molecule of coenzyme A to set free the first pair of fatty acid carbons and to yield a new, shortened acyl coenzyme A.

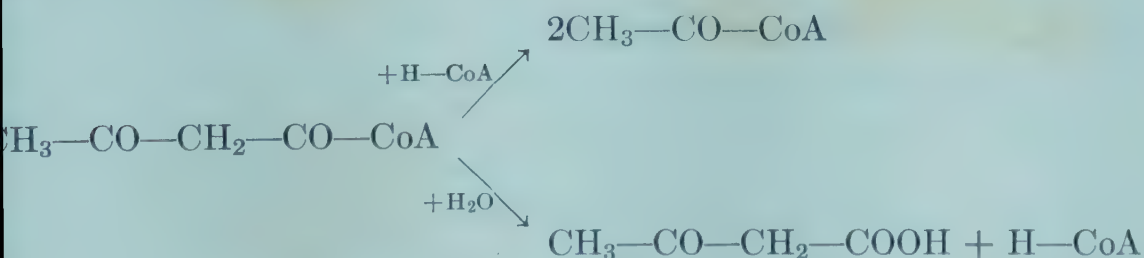


By repetitions of the last two steps the new acyl CoA may be progressively degraded to acetyl coenzyme A. Meantime, some of the acetylated fragments recondense to form acetoacetate and to set free the coenzyme.



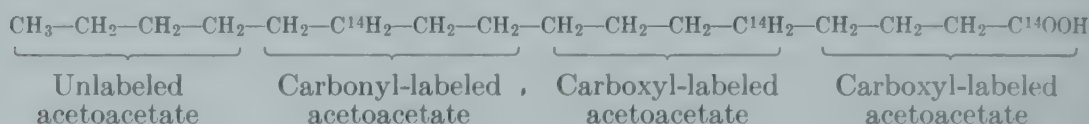
We shall see later that the acetylated coenzyme may follow other paths, but for the moment we are chiefly concerned to evolve a mechanism which will explain the known facts of ketogenesis from fatty acids.

It must be recognized that some small part of the acetoacetate may arise as Knoop suggested from the terminal carbon atoms of a fatty acid. The sequence of reactions outlined by Green would lead to the formation finally of acetoacetyl coenzyme A. This may react as have the other acyl complexes, and set free two final molecules of acetyl coenzyme A. But it might, by a simple hydrolysis, become acetoacetate, and this possibility has not yet been ruled out.



The experimental results which lend most support to the theory of oxidation followed by condensation are as follows. Palmitic acid labeled with radioactive C^{14} at positions 1, 5, and 11 was administered to rats in the form of tripalmitin. Part of the isotope appeared as carbon dioxide and part in acetoacetate, but the distribution of radioactivity in the latter could not possibly be reconciled with Knoop's original formulation or with

others which had attempted to explain formation of acetoacetate as splitting out of four carbons at a time. As indicated in the formula, if Knoop had been right there should have been no radioactivity in the acetoacetate, since the four terminal carbons of the acid are unlabeled. If the molecule had broken into four-carbon fragments each with a β -keto group, no molecule of acetoacetate should have had more than one radioactive carbon, and there should have been twice as many radioactive carboxyl groups as radioactive carbonyl groups.



Actually the radioactivity was randomly and fairly evenly distributed between the carboxyl and the carbonyl groups. The best explanation for this is that two-carbon fragments have broken from the acid chain and that some of these have then recombined at random to form acetoacetate. This theory has the advantage that it explains how the six-carbon caproic acid, yielding three acetylated coenzyme molecules, can give rise to more acetoacetate than butyric acid, which splits to yield only two such fragments. Furthermore it indicates how the odd-numbered acids could also be precursors of acetoacetate through the two-carbon units to which they would give rise. It is probable that the terminal three-carbon acid which would be formed by successive β -oxidations of an odd-numbered acid is not further oxidized but is used as are several other three-carbon compounds in synthesis of liver glycogen. In fact propionic acid has been shown in the intact animal to be a precursor of glycogen rather than of ketone bodies.

To summarize: The oxidation of the fatty acids begins with the removal in sequence of two-carbon fragments which form only in the presence of coenzyme A and which appear condensed with this coenzyme.

Depending on conditions, varying fractions of the acetylated coenzyme molecules recondense to form acetoacetate, setting free the coenzyme.

As noted above, most tissues except the liver can use acetoacetate as an oxidative substrate and it is normally distributed by the blood and used by them. Most of the acetylated coenzyme molecules are oxidized to carbon dioxide and water and the coenzyme is regenerated. The reactions involved in this process are considered in Chapter 14.

DESATURATION OF FAT ACIDS

The formation of the unsaturated palmitoleic acid (see p. 390) from palmitic acid in Schoenheimer's isotope experiments indicates that the

body is able to remove hydrogens from a fatty acid chain. This result was unexpected for it had long been known that certain unsaturated fatty acids are essential dietary factors. The presence of either linoleic, linolenic, or arachidonic acid satisfies the requirement, but many other unsaturated acids do not. This indicates that these compounds, which are required in very small amounts, probably have an arrangement of double bonds which the body cannot duplicate and which fits them to fulfill some vitamin-like function. Schoenheimer's results show that the body does not lack the ability to desaturate fatty acids, but it must be unable to introduce just this necessary pattern of unsaturation.

Early experiments in which the iodine number of food fats was compared with that of liver fats indicated that the liver possesses the ability to desaturate fatty acids. More recently there has been separated from liver tissue an enzyme which dehydrogenates long chain saturated acids. Since isotope experiments have shown that the intact animal can also form saturated acids from labeled unsaturated acids, this reaction, like most other biological reactions, is reversible. Up to the present the dehydrogenase which catalyzes desaturation has been found only in the liver.

METABOLISM OF THE PHOSPHOLIPIDS

The most abundant of the phospholipids is lecithin or phosphocholine, but the group includes also "cephalin,"² several different phosphoinositol derivatives, and more than one compound containing the base sphingomyelin. In general the phospholipids are likely to include a higher proportion of unsaturated acids than is found in the simple triglycerides. The distribution of some of the compound lipids in various plasmas is given in table 12-III, where it will be seen that phosphocholine is by far the most abundant in animal tissues.

Phospholipid Synthesis. The synthesis of the compound lipids in the body has been investigated with isotopic P^{32} and C^{14} and also with a long chain acid known as *elaidic acid*. This compound is the *trans* isomer of ordinary oleic acid, which has the *cis* configuration at its one double bond. It is formed when olive oil, with its high content of oleic acid, is treated with N_2O_3 . Under these conditions about 85 per cent of the oleic acid in the oil is transformed into the geometrical isomer. The insolubility in alcohol of the lead salt of this compound makes it possible to separate and identify it readily and so it has served as a type of labeled molecule in a good many studies of fat metabolism.

² It will be recalled that the phospholipid fraction to which this name was originally given has already yielded four or five different components. The first of these to be identified was phosphoethanolamine, and so if the old name is to be preserved it could probably be applied to this compound.

TABLE 12-III. AVERAGE LIPID PHOSPHORUS, NITROGEN, CHOLINE, AND SPHINGOSINE IN BLOOD PLASMA OF NORMAL ANIMALS ^a

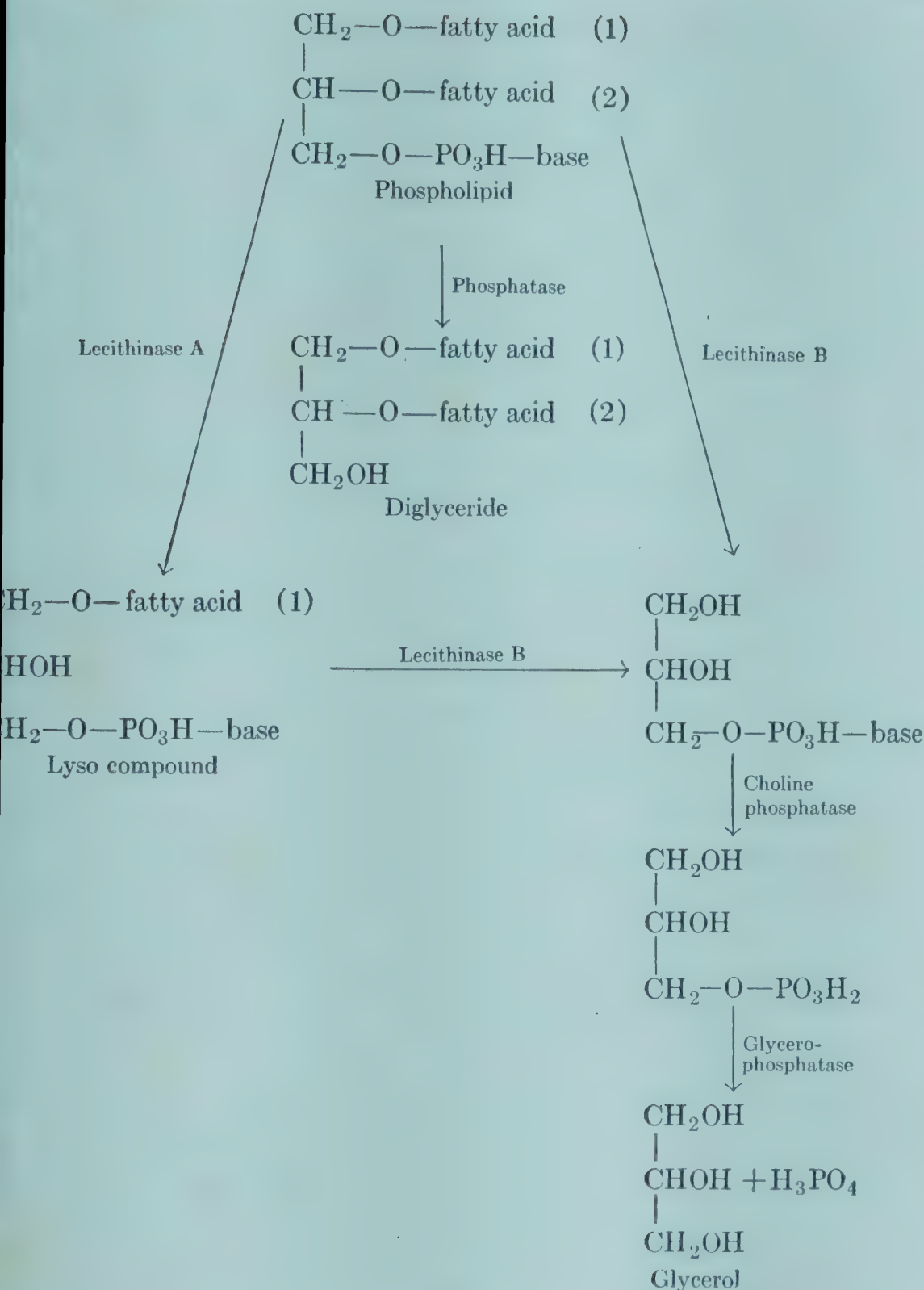
	Lipid P (μ M)	Lipid N (μ M)	Lipid Choline N		Lipid Sphingosine N	
			(μ M)	(% total lipid N)	(μ M)	(% total lipid N)
Rabbit	107	120	90.3	75	21.4	17.7
Rat	129	150	116	77.5	23.8	16.0
Human A	224	288	199	69.1	60.1	20.9
Human B	240	304	228	74.6	56.9	18.7
Human C	256	324	248	76.7	52.9	16.5
Human D	246	314	242	77.0	53.0	16.9
Human E	190	248	182	73.6	46.9	18.9

Values given in micromoles (moles^{-6}) per 100 ml. of blood plasma.

^a Data from W. E. Taylor and J. M. McKibbin, "The Blood Plasma Lipids of Several Species," *J.B.C.*, 188:677, 1951.

Measurements of the turnover rate of the phospholipids in the presence of elaidic acid or of other labeled acids indicate that the compound lipids are synthesized rapidly from dietary fat acids. This synthesis proceeds most rapidly in the liver and the intestine, but takes place at a slower rate in muscle, kidney, and lungs. Nervous tissue and especially brain tissue on the other hand seems not to use preformed fatty acids at all, but to synthesize its own acids from small molecules and body water. Since isotopic phosphate does not appear in plasma phospholipids in hepatectomized animals, it is believed that the liver is the sole source of the compound lipids of the plasma, and that those which are formed in other organs than the liver are not released to the circulation.

Degradation of the Phospholipids. Almost nothing is known of the intermediary metabolism of the compound lipids. There are enzymes in both plant and animal tissue which attack various linkages and which together could achieve complete hydrolysis of either lecithin or of phosphoethanolamine. The two lecithinases, one of which is a simple lipase, have a fairly wide distribution, but their chief interest arises from the presence in certain snake venoms of lecithinase A. The lysolecithin which results from the removal of one fatty acid residue from lecithin has a strong hemolytic effect, that is it causes destruction of both red and white blood cells. Since no such effects attend the normal metabolism of phospholipids in the body, either both fatty acid residues are removed simultaneously or, perhaps, the base is set free before any other hydrolysis takes place. However the degradation of these compounds is initiated, it is reasonable to assume that the fatty acids and the glycerol which they contain enter the metabolic pool of these substances and are metabolized through the ordinary channels.



Of the bases which occur in the phospholipids nothing is known with the exception of a few scraps of information about choline. When the transmethylation reaction was discovered it was assumed that the body could not synthesize methyl groups and that it was an essential function of methionine to provide these groups for the biosynthesis of choline. This is probably true in some degree, but it is now known that the body is also

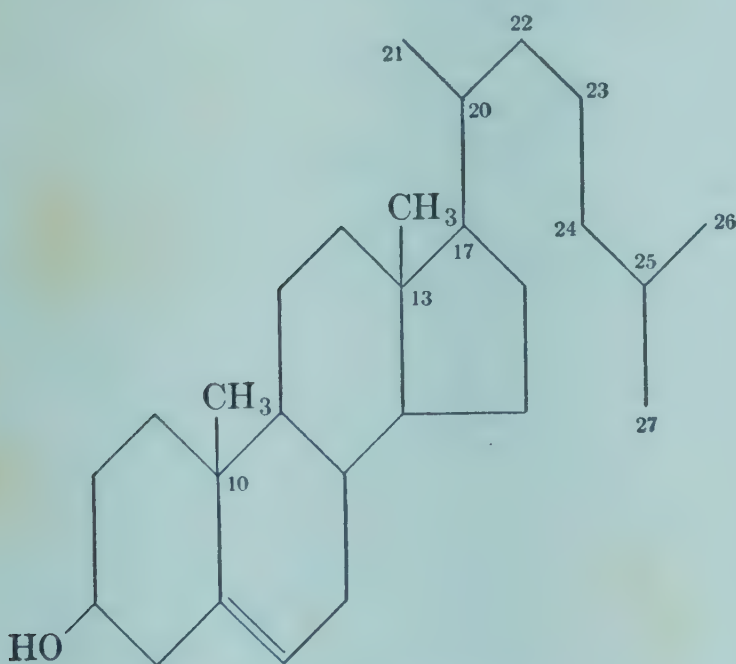
capable of synthesis of these groups from various precursors and that these newly formed groups appear in choline. It may well be that with both methods available body choline normally acquires its methyl groups by transmethylation, but can make use of the relatively slow synthetic pathway when methionine is lacking.

Functions of the Phospholipids. In all cells the phospholipids are major constituents of that constant fraction of the body lipids which cannot be called upon to satisfy metabolic needs. For this reason it is believed that they are essential parts of the living mechanism, and indeed they are known to make up a high proportion of the total lipids of such structural elements as the nucleus and the mitochondria of the cells. Since these structural phospholipids are not readily extracted by fat solvents unless the tissues have been previously hydrolyzed, they probably exist in the cells in lipoprotein complexes similar to those which are present in plasma.

In addition to this structural function two related metabolic functions have been ascribed from time to time to the phospholipids. It has been suggested that they act as vehicles for the transport of fatty acids, both from the fat depots to the liver and from the lumen of the gut into the lacteals during absorption. For years this supposed function has been debated and neither proved nor disproved. A second suggestion for a possible role of the phospholipids in metabolism is that the fatty acids are incorporated into these compounds as a preliminary step in their oxidation. This idea arose originally because of the high degree of unsaturation found in the fatty acids of the compound lipids, which were thus assumed to begin the breakdown of fatty acids by a desaturation. Since it is now known that the desaturation of fat acids is preceded by formation of an acyl coenzyme A complex, it seems unlikely that the phospholipids actually have such a function. Nevertheless attempts are still made from time to time to substantiate the general idea by measuring the turnover rate of dietary acids. If every acid in process of oxidation has to go through a phospholipid stage the dietary acids should be promptly incorporated in the phospholipids and this should be evidenced by a very rapid turnover. The conclusion from many such experiments is that the measured rate in most tissues with the exception of the liver is too low to lend support to this theory of phospholipid function. Thus for the present there is no clearly defined function other than the structural one known for these compounds. Yet their wide distribution and the way in which their concentration in the blood increases at times of active fat metabolism indicate that they probably do play a significant part in the process somewhere.

STEROID METABOLISM

The animal steroids consist, in addition to cholesterol, of a number of compounds with closely related structures. These include the bile acids (see p. 325) and several of the hormones. Among the latter are the hormones of the adrenal cortex and the group of compounds known as the sex hormones which arise in the gonads. In the female the chief site of formation of the sex hormones is the ovary, in which are elaborated compounds which control the cyclical changes in the uterus and others which are concerned primarily with the implantation and nourishment of the fetus. The substances which stimulate the development of the secondary sex characteristics in the male are known as androgens and are formed chiefly in the testes. These compounds all have the same basic ring skeleton, but differ from each other and from cholesterol in the number and position of unsaturated bonds, in the type of side chain at position 17 and in the number and nature of hydroxyl and/or keto groups. Most of them, like cholesterol, have methyl groups at positions 10 and 13 and oxygen either as OH or as a keto group at carbon 3.

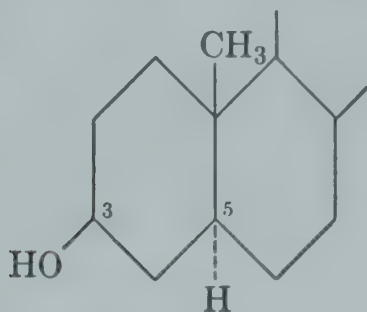


Cholesterol

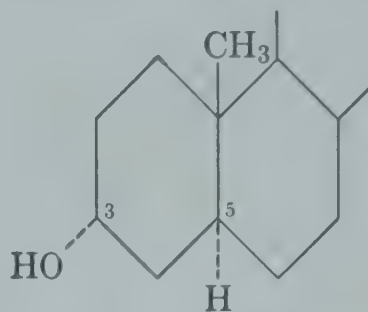
The chemistry of the steroids is extremely complex, particularly because of the possibilities for stereoisomerism. Thus in most of them carbons 3, 5, 8, 9, 10, 13, and 14 are all asymmetric so that the possible number of isomers is overwhelming. Happily, however, only a few of the possible forms occur naturally.

If we assume, not quite correctly, that the ring system lies in the plane of the paper then the H and the OH attached to carbon 3 may extend either

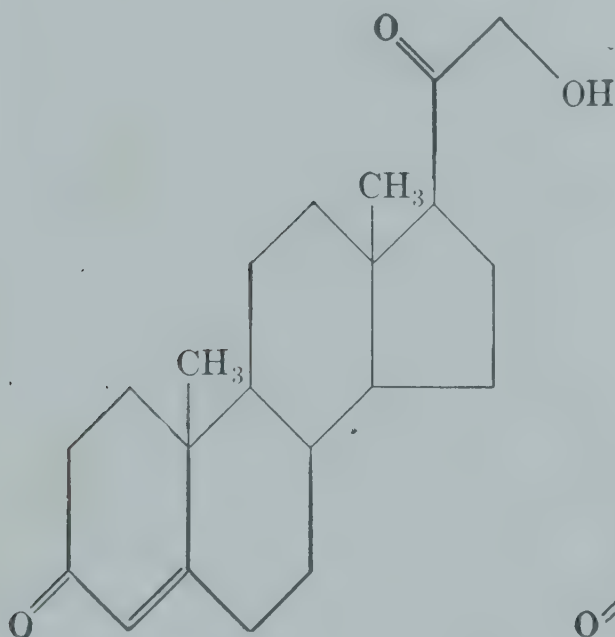
above or below this plane. By convention when the OH group on carbon 3 bears the *trans* relationship to the hydrogen on carbon 5 the OH bond is written as a solid line and that of the H as a dotted line. When these two are in the *cis* relationship both lines are dotted. The *trans* arrangement, also known as the β -arrangement, is characteristic of all the natural sterols.



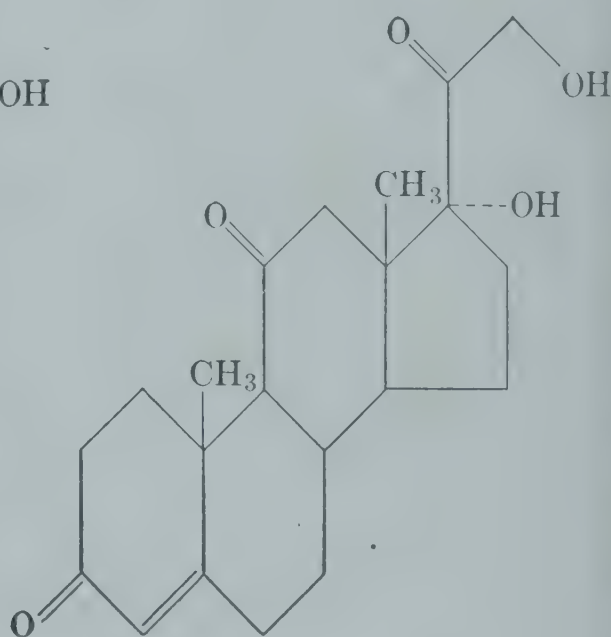
Dihydrocholesterol

*Epidihydrocholesterol*

Six different active steroid compounds have been isolated from the adrenal cortex, all of them closely related to desoxycorticosterone. The dramatic curative effect upon arthritis of the one of these which is named cortisone gives it a special interest.



Desoxycorticosterone

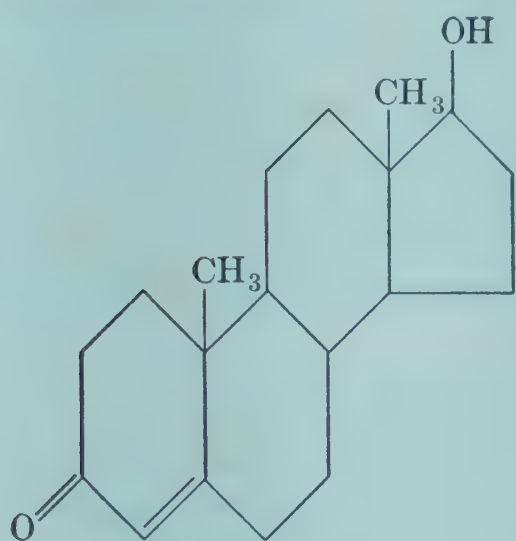


"Cortisone"

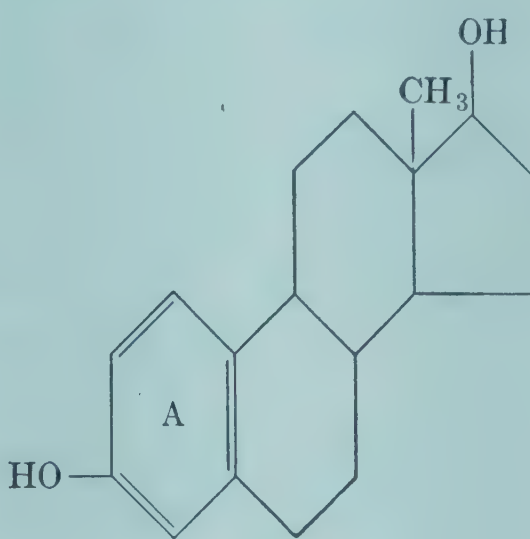
The structural variations found in the other cortical hormones include the presence of an hydroxyl or a keto group at carbon 11 and an additional hydroxyl group at carbon 17. This last is present in cortisone and proves to be *trans* to the methyl group at carbon 13. It is consequently shown in the formula for cortisone with a dotted bond.

The sex hormones are a more varied group, but their structural relationship to cholesterol is still marked. Progesterone is chemically most clearly

related to the adrenal hormones, being identical with desoxycorticosterone except that it lacks the hydroxyl group on carbon 21. The other sex hormones have no side chain at carbon 17 and several of the female ones have an aromatic Ring A. The formulas for testosterone, the most potent of the androgens, and of estradiol, one of the primary hormones concerned with the development of femaleness, will serve to illustrate these variations.



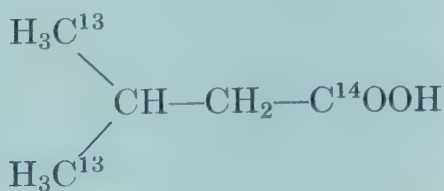
Testosterone



Estradiol

Biosynthesis of Cholesterol. Cholesterol occurs in all animal cells, though it is particularly abundant in brain and nerve tissue. Both in the cells and in the plasma it is found partly in the free condition and partly esterified with fat acids which are usually unsaturated. Of the total steroids of the body, cholesterol makes up approximately 98 per cent.

Although some foods and notably egg yolk do contain cholesterol they do not provide enough to account for the amounts which are found in animal tissues. Thus it has long been clear that the body can synthesize cholesterol and it has been known too that the liver is the chief site of this synthesis. Work with isotopic labels has shown that acetate is an effective precursor and indeed can probably furnish a high proportion of all the carbons which are needed. Here again is a two-carbon unit used in synthesis, but there is also evidence that in the fashioning of the complex steroid skeleton some larger units may participate without first being degraded to acetate. Thus administration of doubly labeled isovaleric acid gives rise to cholesterol in which it appears that the carbons of the



Isovaleric acid

isopropyl group of that acid have been incorporated as a unit. Other simple units which may participate in the synthesis are $C^{14}O_2$ and the deuterium of heavy water. The information presently available does not allow any real definition of the course of cholesterol synthesis, though there is some evidence that acetoacetate is an intermediate. Determination of the exact place taken by the carbon and hydrogen atoms of each precursor must await further experimentation.

Steroid Degradation. The major part of the cholesterol of the plasma and the organs is synthesized in the animal, chiefly by the liver but probably in limited amounts in most other organs as well. The small amount which is of dietary origin is absorbed with other lipid material into the lacteals and in the course of this process about half of it is esterified with fatty acids. Injected cholesterol is removed rapidly from the circulation, going chiefly into the liver. Thus the liver steroids are apparently in equilibrium with those of the tissues and the plasma.

Although it is a tempting theory, there is at present very little evidence that the abundant supply of cholesterol in the body is used in the synthesis of steroid hormones. Indeed recent evidence seems to point in the opposite direction, and to indicate that cholesterol is not an obligatory intermediate in hormone synthesis. Since both the hormones and cholesterol can be synthesized from acetate, this probably means that there is at some point a branching in the synthetic pathway, and that the molecules which follow one path become cholesterol and those which follow the other give rise to the hormones. This does not of course rule out the possibility that transformation of cholesterol is an alternative method of preparing hormone molecules.

Of other possible metabolic transformations of cholesterol nothing is known beyond the fact that some of it does "disappear" when labeled cholesterol is injected into an animal. To what extent this difference between the amount injected and the amount which later can be accounted for in excreta and carcass is due to oxidation is not known. It has been shown that carbon 26 of the side chain is readily oxidized and that a labeled carbon in the ring is attacked very much more slowly, but whether this process ever goes on to complete oxidation and destruction of the rings is still a question.

There are several pathways by which cholesterol is excreted. Some of it appears unchanged in the bile where it is normally held in solution by the bile salts and so carried into the feces when the bile is discharged into the upper intestine. When for any reason the amount of cholesterol in the bile becomes too great to be held in solution it is deposited in the gall bladder as gallstones, many of which are nearly pure cholesterol. Some of the tissue cholesterol is transformed into the saturated dihydrocholes-

terol and is then secreted directly into the large bowel for excretion. The third sterol which is found in the feces is coprosterol. This is an isomer of dihydrocholesterol with a different stereochemical configuration at the point of attachment of rings A and B to each other. It is therefore believed that formation of coprosterol does not involve a simple reduction, which gives rise to dihydrocholesterol, but that cholesterol undergoes in the bowel a series of oxidative reactions in the course of which a symmetrical intermediate is formed which makes possible the new stereoisomer. The reactions, whatever they are, apparently take place in the colon, since coprosterol is never found in the tissues.

The main vehicle of excretion of the steroid hormones as distinct from cholesterol is the urine, from which a large and confusing variety of derivatives has been obtained. Their interrelationships and the conditions which lead to their excretion in large amounts are outside the scope of this book.

Plant Lipids

Although some lipids occur in all portions of the plant, since they make up part of the essential structures of all living cells, they are found in high concentrations only in the fruit and seeds. The seeds of most families of higher plants except the cereals contain fat as reserve food, often to the exclusion of starch.

LIPID EQUILIBRIUM IN PLANTS

There is evidence that in plants as in animals the fats and carbohydrates are part of a mobile equilibrium. For example it has been shown with several species that sugars disappear from seeds during the period of fat accumulation, which process goes on very rapidly at the time when the seeds are ripening. Furthermore, measurement of the gas exchange has shown that the respiratory quotient of the seeds during this period is well above 1 but falls to a much lower value when the rate of fat accumulation decreases. Thus the high R.Q. is believed to indicate formation of the oxygen-poor fat from carbohydrates with their relatively high oxygen content.

Of the pathway by which the transformation of carbohydrate to fat is achieved in plants but little is known. It has been shown that in the developing flaxseed free fatty acids appear in relatively high concentration before the concentration of triglycerides increases appreciably. This points to a preliminary accumulation of fat acid stores. It is probable that in the plant as in the animal these fat acids are synthesized from small molecules formed in the course of carbohydrate metabolism. There is evidence also that formation of saturated acids precedes formation of

unsaturated ones, since during ripening of seeds the iodine number rises rapidly. This may mean a desaturation of the acids previously formed, or it may indicate a preferential use of saturated acids for the metabolic needs of the seed. Among the unsaturated acids formed in plants are the linoleic and linolenic acids which are essential ingredients of animal diets.

In the plant the reverse process of carbohydrate synthesis from fat seems to take place when the seeds germinate. Here again there are two lines of evidence. The fats disappear from the germinating seeds at the time when the essentially carbohydrate framework of the embryo is being formed. With some species there is also an actual appearance of sugar in the seedling as the fat disappears. Furthermore the gas exchange of the whole castor bean seedling at this time gives an R.Q. of about 0.3. This can only indicate a process in which fat is being converted to something far richer in oxygen so that oxygen is being used with no corresponding evolution of carbon dioxide. For example, if the ricinoleic acid which is the major constituent of castor bean fat were being entirely converted to sucrose the process would have an R.Q. of zero!

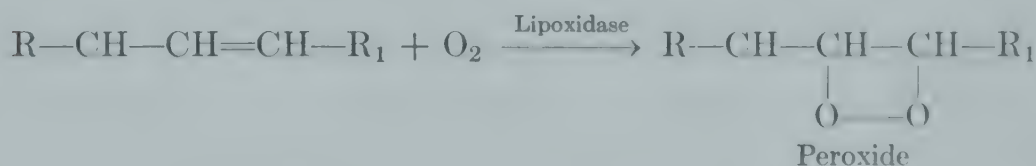


When the endosperm which contains the seed fat is removed from the growing embryo, the R.Q. of the latter is found to be very nearly 1. This indicates that the chief metabolite of the actively growing embryo is carbohydrate which must have been derived from the stored fat of the endosperm.

So far as we now know then the plant uses fats much as the animal does to provide an efficient reserve energy store which is chiefly laid down in the seeds. Plants and animals both use the products of fat metabolism to synthesize carbohydrates, and the small molecules formed in carbohydrate metabolism to synthesize fat acids.

Oxidation of the Fats. Presumably in the plant as in the animal fat metabolism begins with hydrolysis since all plants are well supplied with lipases. As might be expected these enzymes are especially abundant in seeds with a high fat content.

The only known plant enzyme specifically concerned with oxidation of fat acids is *lipoxidase* which causes the highly unsaturated linoleic, linolenic, and arachidonic acids to form peroxides at the double bonds.



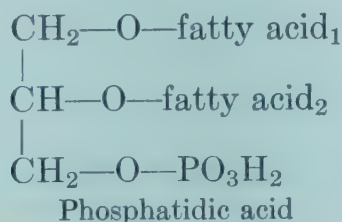
The enzyme is inactive with oleic acid and also with any except the natural isomers of the unsaturated acids on which it does act. For example it has

no effect on the *trans* isomers of linoleic or linolenic acids nor on isomeric acids in which the double bonds are conjugated.



Since peroxides are strong oxidizing agents, the ones formed in the plant are able to bring about nonenzymic oxidations and are responsible for the oxidative destruction of carotene in stored vegetables. But whether or not peroxide formation is part of the normal oxidative use of unsaturated fat acids is not known. It seems likely that fatty acid breakdown in the plant follows the pathway of β -oxidation, setting free two-carbon units which are then either oxidized completely or used in synthesis. The search for such a system in plants has not yet been made.

Plant Phospholipids. The compound lipids of plants are found in small amounts in all cells. They include besides lecithin and phosphoethanolamine, various phosphatidic acids, and a group of compounds known as *lipositols* in which the cyclic hexahydric alcohol, inositol, is present in place

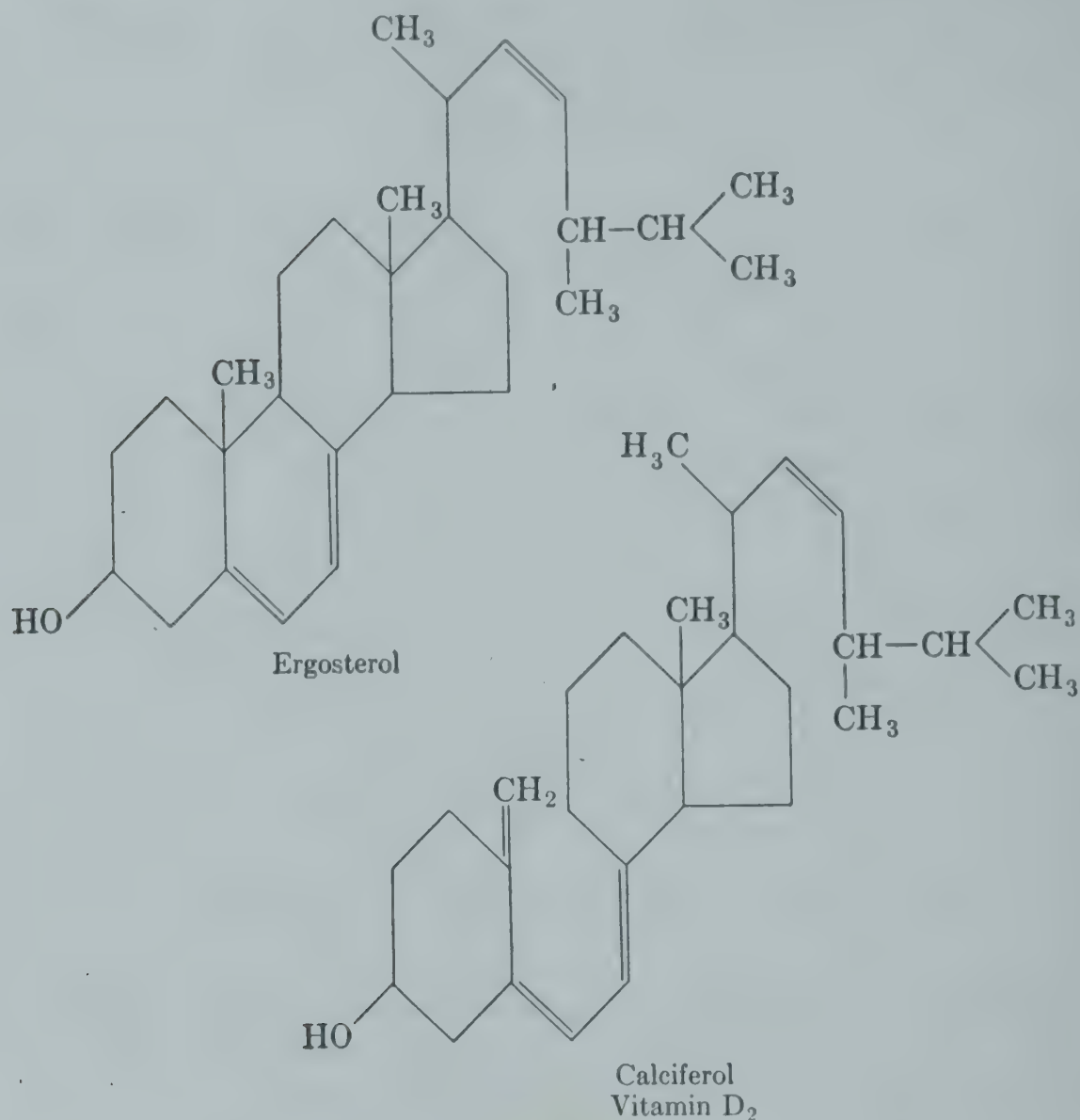


of glycerol. In some plant cells at least the lipositols make up a much larger fraction of the total phospholipids than in animal cells. The structure of the lipositols has not been worked out.

Of the metabolism and function of the plant phospholipids almost nothing is known. Some of the enzymes which hydrolyze the various bonds in lecithin have been identified in a few plant tissues, but no comprehensive picture of their action has emerged. There is some evidence that the phospholipids of plants are, like those of animals, important structural units in the cells. Thus, for example, the phospholipids of the leaf seem to be concentrated in the chloroplasts as in animal cells they make up a large part of the lipid fraction of cell nuclei and mitochondria. In both types of tissue the phospholipids seem to be part of a still larger lipoprotein complex.

Plant Sterols. Although it was first isolated from ergot, a fungus which infects various grains, ergosterol also occurs in low concentrations in many plants. This is indicated by the fact that ultraviolet irradiation of these

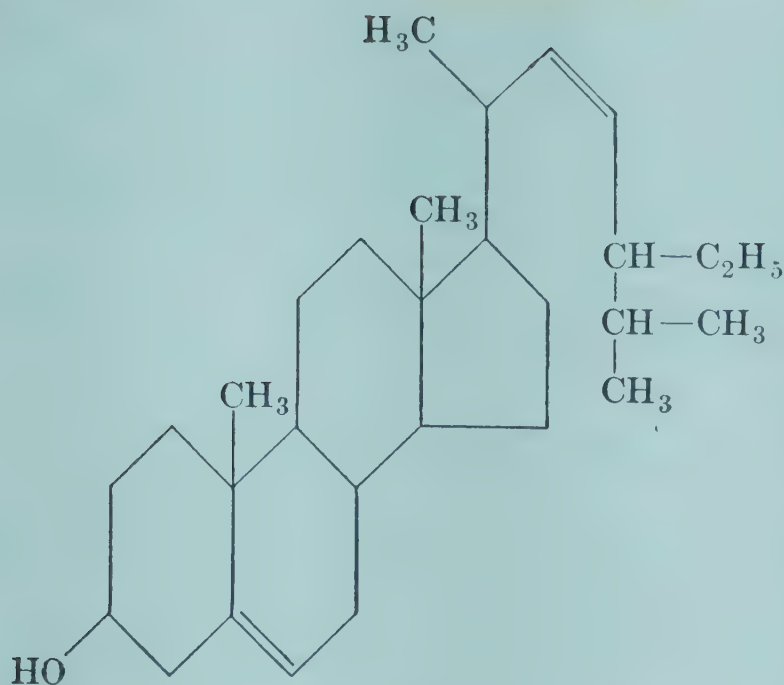
plants gives rise to calciferol which is the compound responsible for the antirachitic potency of irradiated foods.



As indicated in the formulas, radiation breaks ring B of the sterol and transforms the methyl group at carbon 10 into a doubly bound methylene group.

Except for ergosterol the plant sterols, or *phytosterols*, are not well characterized. They include a group of derivatives with a ten-carbon side chain at carbon 17, known as *sitosterols*, of which the stigmasterol of soybean and calabar bean oil is the only one for which a formula can be written at the present time.

The concentration of all these compounds in plant cells is low and it is not known whether or not they constitute part of the lipoproteins of essential cell structures. In fact almost the whole field of plant lipid metabolism lies open for future investigation.



Stigmasterol

Suggestions for Further Reading

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The *Annual Review of Biochemistry* carries a yearly article on the metabolism of the lipids from which references may be obtained to work on all phases of the subject.

Study Questions

1. Distinguish between "depot" and "structural" lipids. What is the experimental basis for the distinction?
2. Describe the Schoenheimer experiment with labeled palmitic acid.
3. Sketch the general picture of fatty acid metabolism which emerged from the work with isotopically labeled fatty acids and contrast this with the older view.
4. For what two reasons are fats valuable as fuel?
5. What is the "respiratory quotient"? How is measurement of this value used to determine the type of material being oxidized by a cell?
6. What metabolites are known to contribute to fatty acid synthesis in living cells? Are the fatty acids used as precursors of other primary foodstuffs? Give the experimental basis for your answer.
7. What is meant by "fatty liver"? What is a lipotropic agent? Name three.
8. What are the ketone bodies? Write their formulas and indicate their relation to each other.
9. Indicate by formulas the course of the oxidation of palmitic acid according to modern theory, and show two ways in which this can lead to formation of acetoacetate.
10. What was the original evidence for the theory of β -oxidation?

11. What are the "essential" fatty acids? Where are they formed?
12. What is known of the functions of the phospholipids? Of their synthesis?
13. What is known of the biosynthesis of cholesterol?
14. State several specific ways in which the metabolism of fats in plants is believed to follow the same paths as in animal cells. Give the experimental basis for your comparison.

Anaerobic Carbohydrate Metabolism

There is no good evidence that in any of its manifestations life evades the second law of thermodynamics, but in the downward course of the energy-flow it interposes a barrier and dams up a reservoir which provides potential for its own remarkable activities.

F. G. HOPKINS (1933)

In recent years it has become evident that the chemical reactions through which glucose functions as a fundamental oxidative substrate are nearly identical for a wide variety of plant, animal, and microbial cells. The degradation of glucose to carbon dioxide and water involves a series of individual reactions which may be classified for convenience in two groups. As a result of the reactions which constitute the first stage, each molecule of glucose yields two of pyruvate if the cell is metabolizing under normal aerobic conditions. In the second stage the pyruvate is oxidized to carbon dioxide and water. However, if the cell is metabolizing anaerobically, the pyruvate is not oxidized but, depending on the catalysts available, may yield one of several possible derivatives such as lactic acid or ethyl alcohol. It is with the reactions which take place under anaerobic conditions that the present chapter is chiefly concerned. The experimental evidence cited was obtained largely in work with muscle, but the chemical events which this work elucidated are of very nearly universal significance.

Anaerobic Carbohydrate Metabolism of Muscle

Most living organisms with the exception of a small group of bacteria are either strict or facultative aerobes, that is, they either require oxygen in order to survive, or can utilize it when it is available. In view of this fact it may seem strange that it is the anaerobic metabolism of carbohydrates which is first considered here. There are two reasons for this choice. In the first place, this phase of carbohydrate metabolism was investigated long before anything was known of the oxidative mechanisms, and in the course of the last half century a good deal has been learned about it. The other reason is that it is now known that the anaerobic metabolism is not a separate metabolic path, entirely divorced from the oxidative one. It constitutes rather a preparation of the carbohydrate

52,000 cal. per mole of glucose. It is therefore evident that in order to obtain a given amount of energy, fermenting cells will have to degrade far more glucose than would be needed under aerobic conditions.

MUSCLE AS EXPERIMENTAL MATERIAL

As was noted earlier, muscle has much to recommend it as an experimental material. Certain muscles, such as the sartorius or the gastrocnemius muscles, can be removed from the body uninjured and will, when stimulated, perform amounts of work which are easily measurable, and which can be correlated with the attendant chemical changes.

Muscular tissue consists essentially of bundles of fibers made up of elongated cells highly specialized for contractility. Muscle cells contain about 25 per cent of solid matter, of which roughly four fifths is protein. Employing different preliminary treatments, two proteins, *actin* and *myosin*, have been extracted from muscle. In the living tissue these two constitute the major part of the protein content, and there is a good deal of evidence that they are there united as a complex known as *actomyosin*. This substance is believed to be the actual contractile unit in the living muscle cell.

Myosin is a typical elongated fibrous protein and might therefore be expected to have the ability to contract as wool does, through a folding or coiling of its carbon chain. Actually it has never been possible to bring about contraction of purified myosin.

Actin makes up 15–20 per cent of the total muscle protein and as ordinarily isolated consists of globular molecules known as G-actin. When small amounts of salts are added to its solution, profound and highly anomalous changes take place in its physical properties. These are believed to result from its transformation into a fibrous form. Hence in this state it is known as F-actin. If myosin is added to the solution of F-actin the highly viscous actomyosin results, and this complex does undergo contraction under certain conditions. The ultimate objective in the study of muscle metabolism is the elucidation of the linking of this physical change with the chemical events which accompany or induce it.

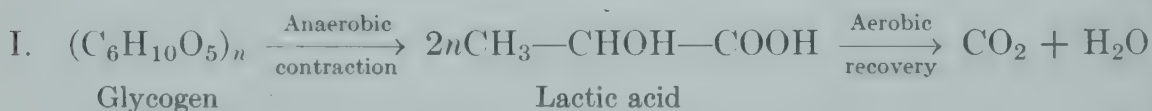
EARLY WORK ON MUSCULAR CONTRACTION

It had long been known that lactic acid is formed in muscles, but at the turn of the century there was much conflicting evidence both as to the amount of the acid in resting muscle, and as to its relation to contraction. In 1907 Fletcher and Hopkins at Cambridge published the now classical paper which put the study of muscle chemistry on a firm basis. They used frog muscles in pairs, stimulating one and keeping the other under otherwise identical conditions as a control. At the end of an experiment the muscles were suddenly chilled by immersion in ice-cold alcohol. This

killed the tissue enzymes so that the subsequent grinding with sand did not give rise to enzymatic changes unrelated to the experiment in hand. Analysis of the alcohol extracts from the paired muscles then showed what chemical changes had taken place as a result of the stimulation. The results were clear cut and may be summarized as follows:

1. Resting muscle contains only traces of lactic acid.
2. Muscle can contract in an atmosphere of nitrogen, i.e., in complete absence of oxygen.
3. During these anaerobic contractions, lactic acid forms in increasing amounts, reaching a maximum when the fatigued muscle is no longer responsive to stimulation.
4. When a fatigued muscle is allowed to rest in an atmosphere of oxygen, it recovers its irritability, and the lactic acid simultaneously disappears.
5. Stimulation of a muscle in oxygen gives rise to less lactic acid than is formed anaerobically.

In 1920 came the first of Meyerhof's¹ long series of contributions to muscle chemistry. He showed that during stimulation glycogen disappears in amounts equivalent to the lactic acid which forms. At this time the chemical events in muscle might have been outlined in some such scheme as the following:

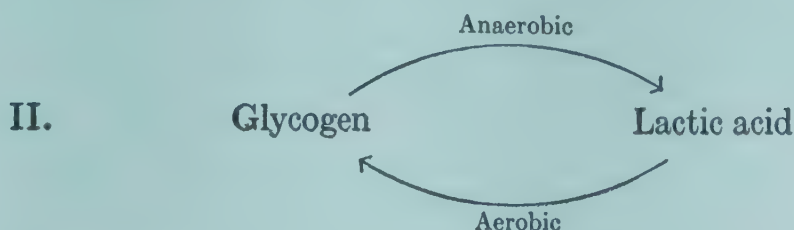


It was believed that the first step was the crucial one in which degradation of glycogen to lactic acid furnished the energy for the contraction.

In later quantitative experiments with frog muscles Meyerhof measured the oxygen-uptake during recovery. He found, to everyone's surprise, that the amount of oxygen used was only about one quarter to one fifth of that which would have been needed to oxidize all the lactic acid which disappeared. Furthermore, during recovery the glycogen stores which had been depleted by contraction were reconstituted. From these results Meyerhof concluded that most or all of the lactic acid formed in the muscle during contraction is resynthesized to glycogen during recovery, the necessary energy for this synthesis being obtained by oxidation of something. Since the R.Q. of the reaction was about 1, the "something" might equally

¹ Otto Meyerhof (1884–1951) had had a brilliant career in biochemistry at Berlin and Heidelberg before political events in Germany forced him to seek what proved to be only a temporary refuge as Director of Biochemical Research at the University of Paris. The coming of the Germans in 1940 again drove him into exile, by which biochemistry in the United States has been greatly enriched. As Research Professor of Physiological Chemistry in the School of Medicine of the University of Pennsylvania he directed an active research program until shortly before his death. In 1923 Meyerhof and A. V. Hill of London shared the Nobel Prize in Medicine, awarded for their contributions to muscle chemistry and physiology.

well have been glucose or part of the lactic acid, and there was no way to choose between them. At this time the chemical changes in frog muscle were formulated according to the following scheme. The energy for con-



traction was still believed to come from the anaerobic formation of lactic acid, while the oxidation was explained as a "rewinding of the clock" in which as much of the lactic acid as possible was again raised to the energy level of glycogen. Muscles were said to go into "oxygen debt" when they formed during prolonged contraction amounts of lactic acid which had later to be removed by oxidation.

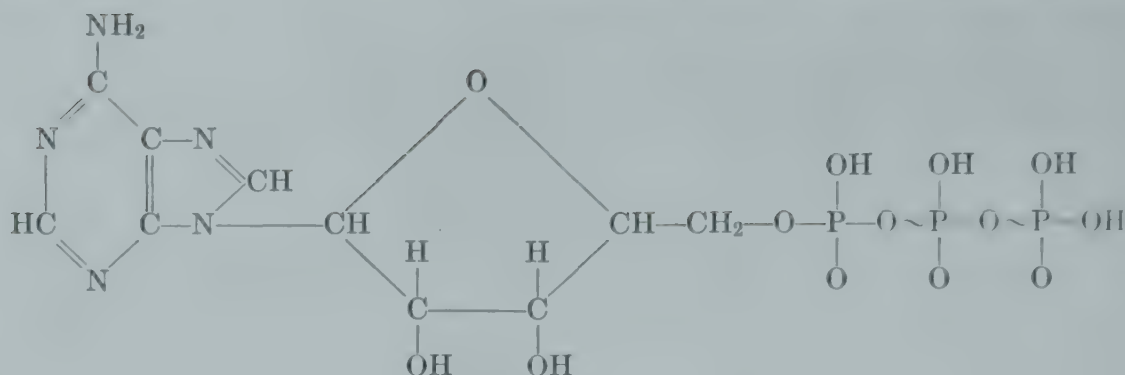
This simple scheme did not prove to be an accurate description of events in the muscles of higher vertebrates. But it was adapted to their needs by the discovery that in these animals most of the lactic acid formed during contraction diffused into the blood and so was carried back to the liver and there synthesized into glycogen. Thus ultimately the lactic acid might be considered a source of fresh stores of muscle glycogen, since the liver glycogen would furnish glucose to the blood and this in turn might go into the muscle cells as muscle glycogen.

MUSCLE EXTRACTS

An enormous impetus was given to progress in muscle chemistry by Meyerhof's discovery about 1926 that extraction of ice-cold minced muscle with saline yields an opalescent cell-free solution which contains no oxidizing enzymes, but does include all of the enzymes required for the transformation of glycogen to lactic acid. Thus the way was opened for a direct chemical attack upon the problem of the intermediate steps involved in the anaerobic degradation of glycogen.

Dialysis of muscle extract led to its separation into a thermolabile inactive protein part, and what proved ultimately to be a mixture of thermostable coenzymes. Addition of fresh, boiled muscle juice to the inactive protein part restored the activity. By about 1930 Lohmann² had shown that the dialyzate contained two essential ions, Mg^{++} and $H_2PO_4^-$, and a third substance which has in the course of the years proved to hold a very special place in the economy of living things. This is *adenosine triphosphate*, or ATP, a phosphorylated derivative of the simple nucleotide known as adenylic acid or adenosine monophosphate (AMP).

² Karl Lohmann (1898–), at one time a colleague of Meyerhof, is Professor of Physiological Chemistry in Berlin.



Adenine

Adenosine

Adenylic acid or AMP

Adenosine diphosphate or ADP

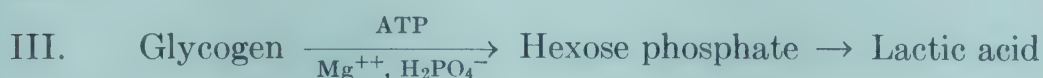
Adenosine triphosphate or ATP

The chief importance of ATP resides in the two oxygen-phosphorus bonds which are represented by the \sim sign. This designation was introduced by Lipmann³ to distinguish certain bonds which he calls "energy rich" from ordinary phosphate ester linkages. When ATP is degraded in such a way that one phosphate group is removed at a time, it is found that rupture of each of the two terminal phosphate bonds makes available about 12,000 cal. per mole, whereas removal of the third phosphate group releases only about 3000 cal. Further investigation of organic phosphate compounds reveals the fact that they can be divided into two distinct groups. In the one the phosphate bonds are energy poor and furnish about 2000-4000 cal. per mole when hydrolyzed. In the other the bonds have roughly the energy content of the terminal bonds of ATP and their rupture makes available 11,000-15,000 cal. per mole. Compounds of the latter type are distinguished by having the ability not only to set free these large amounts of energy, but to transfer it almost intact when they transfer a phosphate group enzymatically to certain other compounds. Both of these properties are utilized by the cells.

It was noted above that addition of Mg^{++} , inorganic phosphate, and ATP to the inactive proteins of dialyzed muscle extract restored its ability to form lactic acid from glycogen. This was not the first hint of the involvement of phosphate in carbohydrate utilization. As early as 1914 Gustav Embden at the University of Frankfurt had isolated a hexose phosphate mixture from muscle and named it "lactacidogen" to indicate that it was a precursor of lactic acid. It was also known that when muscle

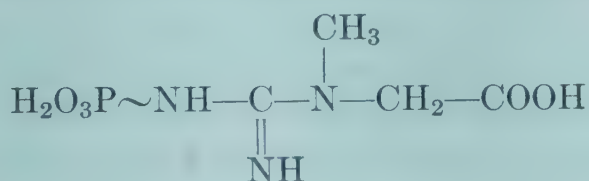
³ Fritz Lipmann (1899-) began his career in Berlin and later spent seven years at the Biological Institute of the Carlsberg Foundation in Copenhagen. He is now Professor of Biological Chemistry at the Harvard Medical School and Director of Biochemical Research at the Massachusetts General Hospital. The concept of the energy rich bond grew out of his work in enzyme chemistry. In 1953 Lipmann shared with H. A. Krebs the Nobel Prize in Medicine.

extract acts upon glycogen the concentration of inorganic phosphate falls, and it was believed that this indicated its conversion to a hexose phosphate. Accordingly the dialyzed extract, inactive toward glycogen, was next tested with hexose phosphate as substrate. Under these conditions the extract was able to catalyze formation of lactic acid. This could only mean that the hexose phosphate was an intermediate product between glycogen and lactic acid, and that dialysis had removed from the extract the factors essential to formation of the hexose phosphate from glycogen. Thus the series of events as understood about 1931 might have been formulated as follows:



IDOACETATE INHIBITION

And then, just when it seemed that the metabolic path from glycogen to lactic acid might be elucidated, came reports of an experiment which made it seem for a time that lactic acid formation had no vital connection with muscular contraction. Einar Lundsgaard of the University of Copenhagen, working with muscles poisoned with the enzyme inhibitor, iodoacetic acid (CH_2ICOOH), found that although they formed no lactic acid they were still able to contract for a short time. Further investigation showed that the length of time during which isolated muscle remained irritable depended upon the concentration in the muscle of a very labile phosphate compound, *creatine phosphate* or phosphagen.



Creatine phosphate

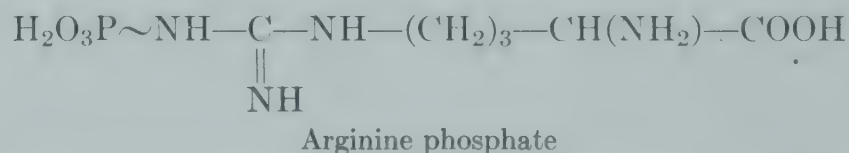
As the formula indicates, this phosphate linkage is of the energy rich type, and it might well therefore furnish the energy for contraction as Lundsgaard suggested. But muscles contain also ATP with its high energy phosphate bonds and this compound was already known to play a part in the carbohydrate metabolism of muscle. For several years there was therefore some disagreement about the roles of these two compounds in muscular contraction. It was the proof of a unique relationship between the muscle protein, myosin, and ATP which led to the present belief that the energy reserves which are called upon at the moment of contraction are probably those of the phosphorylated nucleotide. It has been found that the high energy bonds are freely and reversibly transferable between ATP and phosphocreatine, though in this reaction only the terminal phosphate residue of the triphosphate is transferred, leaving adenosine diphosphate or ADP.



It is now believed that when the need arises for energy for contraction it is furnished by rupture of the terminal phosphate bond of ATP. At this point the presence of a mobile reserve of high energy phosphate in phosphocreatine makes possible the immediate reconstitution of ATP as indicated in the equation. These two compounds will thus keep the contractile machinery in action until the processes of carbohydrate breakdown in the muscle can begin to generate fresh stores of energy.

The fact, referred to above, which relates ATP in a very special way to contraction is as follows: when highly purified myosin is added to ATP it brings about the hydrolytic removal of the terminal phosphate residue. That is to say, it acts as if myosin were the enzyme adenosine triphosphatase, or ATP-ase. This fact was first noted in 1939 by Engelhardt and Ljubimowa of the Institute of Biochemistry in Moscow, and has since been abundantly confirmed. At the same time there is evidence that ATP exerts a reciprocal influence upon myosin, an influence which brings about an increased coiling of the protein chain. This situation was described by Engelhardt in 1946 as follows: "Myosin can be compared with the piston, and ATP with the explosive mixture of a combustion engine. The ingenuity of nature consists in providing the piston with the properties of the ignition plug as well."

The claim of myosin to be both contracting protein and enzyme protein has not gone unchallenged. Meyerhof, for example, believed that the enzyme is a separate entity adsorbed on the myosin and carried along through the various steps in its purification. But whether they prove to be one substance or two in close association, there is now fairly general agreement that in the living muscle ATP is the substance which initiates the changes in myosin which give rise to contraction. Among the vertebrates the energy needed for the immediate rephosphorylation of ADP is stored as phosphocreatine, but among the invertebrates a similar compound of arginine serves this same purpose. In arginine phosphate there is an energy rich linkage between phosphorus and nitrogen, as there is in



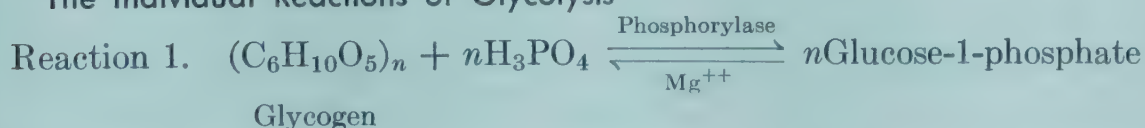
creatine phosphate, and this makes possible a similar transfer of a phosphate group to ADP, with formation of ATP.

THE GLYCOLYTIC SEQUENCE

It would be far too time-consuming to continue to follow historically the slow and sometimes faltering steps by which the complex series of reactions now known as "glycolysis" was finally brought to light. The methods used were standard ones. Enzyme inhibitors were used to cause

one intermediate compound or another to accumulate, as phosphoglyceric acid accumulates in the presence of fluoride, or succinate in the presence of the competitive inhibitor, malonate. In other experiments enzymic reactions were carried out in the presence of a compound which would combine with some intermediate and trap it in a form that could be isolated. This was first done by Carl Neuberg in a study of alcoholic fermentation, when he showed that acetaldehyde was an intermediate by carrying out the fermentation in the presence of sodium bisulfite. The crude muscle extract itself was fractioned again and again by the classical methods of enzyme chemistry until one by one the various specific enzymes had been isolated and concentrated, and many of them crystallized. This all finally led to the realization that at least twelve separate enzymic reactions are involved in the fermentation of glycogen to lactic acid, with as many separate enzymes and a number of coenzymes. The scheme in Figure 13.1 indicates the sequence of the reactions and the points in the series at which ATP is either used or synthesized. When it is being used it appears on the arrow. The net result is synthesis of three high energy bonds for each molecule of glucose metabolized. Actually four new phosphate links are formed, but one of these merely replaces the one which is used early in the sequence. The numbers on the arrows refer to the discussion of the individual steps which follows. It should be noted that biochemists refer rather loosely to a substance as an acid, even when the pH of the medium is such that it is not an acid but its anion which is actually present. This practice is likely to continue as a matter of convenience particularly as several di- and tri-basic acids are involved in metabolism. Phosphoric acid, for example, may be present as any one of three ions, depending on the pH of the solution, and referring to it simply as "phosphate" or as "phosphoric acid" avoids having to be specific in situations in which specificity may be impossible or unimportant.

The Individual Reactions of Glycolysis ⁴



The first reaction in the sequence was one of the last to be discovered. In 1936 the Coris, Carl and Gerty, announced the isolation from frog muscle of a new phosphate ester which appeared to be a monophosphate. Up to this time the only known monophosphates were esterified at position 6, but the new compound differed from these in being nonreducing, and in giving rise to reducing power when it was hydrolyzed. It proved to be a derivative of glucose with the phosphate residue at carbon 1. It is formed in muscle as a result of *phosphorolysis* of glycogen (see p. 244). In this reaction phosphoric acid acts as water does in hydrolysis, breaking

⁴ The metal ions which are written below the arrows in certain of the equations which follow are essential to the catalytic action of the enzyme involved. Their position does not indicate that they catalyze the reversal of the reaction.

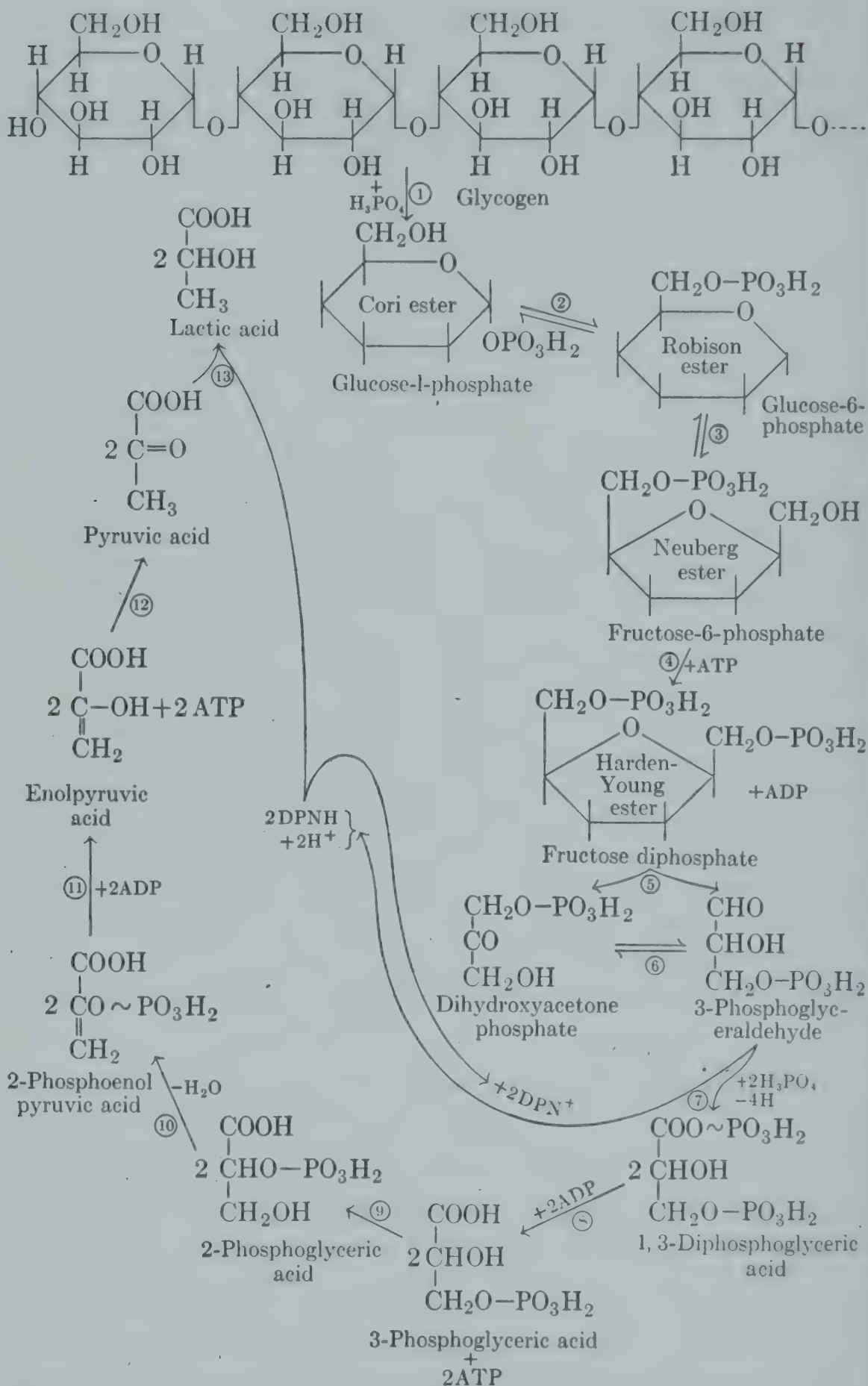
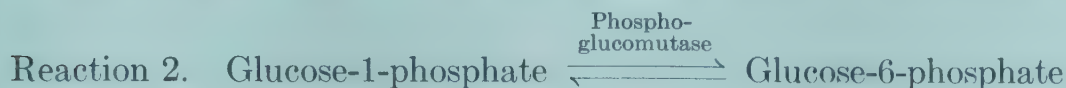


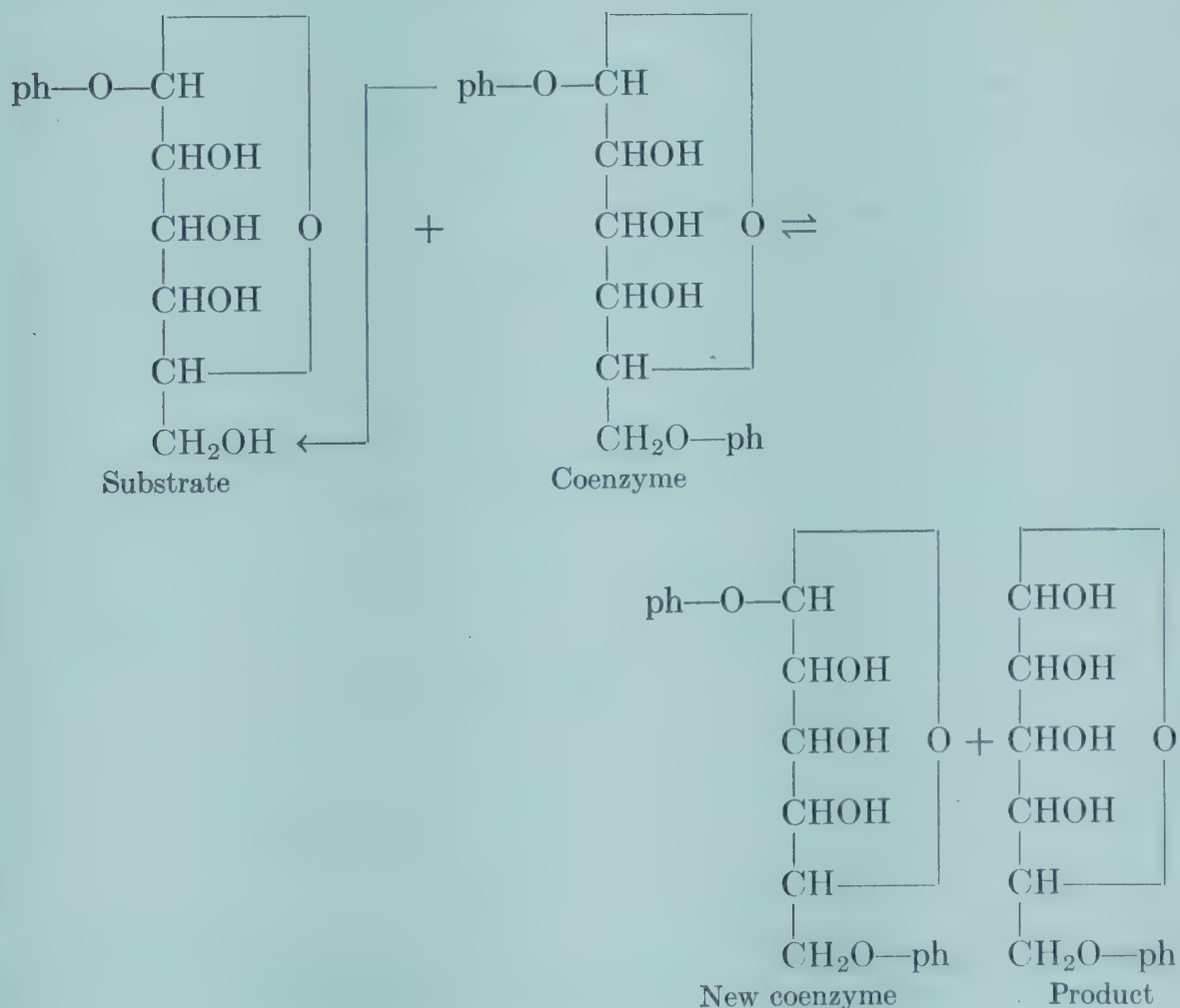
Figure 13.1. The reactions of the glycolytic sequence.

the 1-4' bond, contributing an —OH group to the carbon 4 of one glucose and a phosphate residue to carbon 1 of the other. The *phosphorylase* which catalyzes this degradation has been found in many different animal tissues, while a very similar enzyme from plant tissue brings about phosphorolysis of starch. The enzyme is activated by magnesium ions.



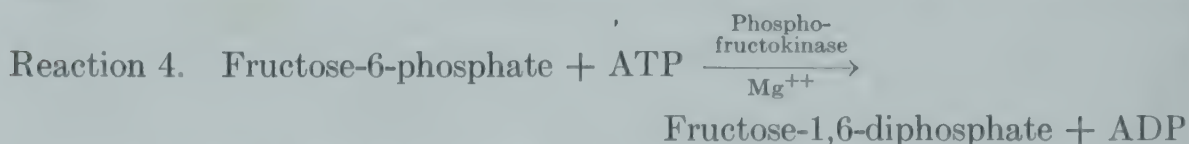
In the presence of *phosphoglucomutase* an equilibrium is established between the two glucose monophosphates. It is strongly in favor of the 6-ester, which makes up nearly 95 per cent of the equilibrium mixture.

Until recently the mechanism of this shift was unknown. It has now been shown that the coenzyme of phosphoglucomutase is α -glucose-1,6-diphosphate. If glucose-1-phosphate is the substrate, the coenzyme acts by transferring to carbon 6 of the substrate the phosphate group from its own carbon 1. Thus it becomes glucose-6-phosphate and forms a new molecule of coenzyme. Any individual glucose phosphate molecule in this way acts in turn as substrate and as coenzyme, the coenzyme of the moment always donating one of its phosphate groups to form whichever of the mono-esters is demanded by the equilibrium. This is indicated in the following scheme, in which "ph" stands for the phosphate residue.



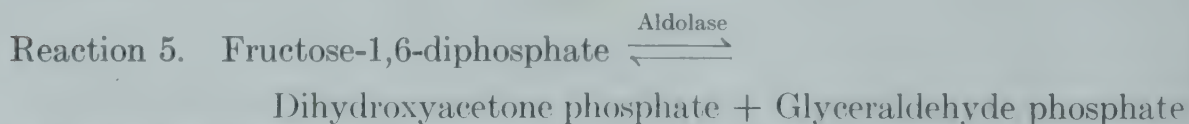


The enzyme which catalyzes this shift is also known as *hexosephosphate isomerase*. It is found closely associated with the phosphoglucomutase of Reaction 2, and with a triosephosphate isomerase (see Reaction 6). Nothing is known of the mechanism of this isomerization except that in the presence of the specific enzyme an equilibrium mixture of the two monophosphates is rapidly formed. As indicated on the chart, glucose-6-phosphate is the Robison ester, fructose-6-phosphate is the Neuberg ester, and the equilibrium mixture of the two is known as the Embden ester. It consists of about 70 per cent Robison ester and 30 per cent Neuberg ester.

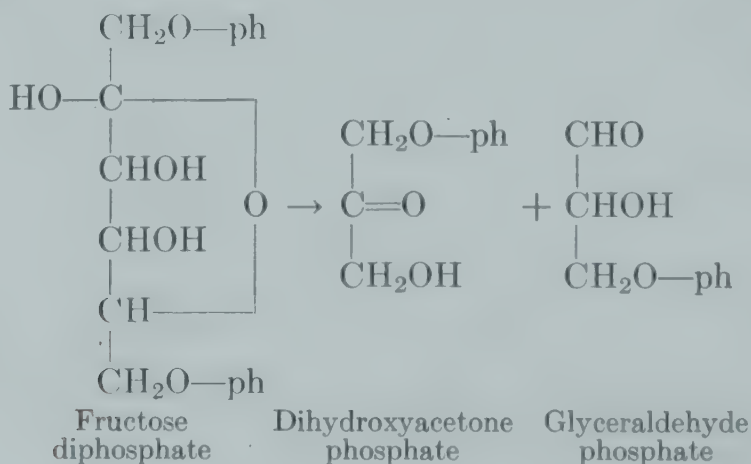


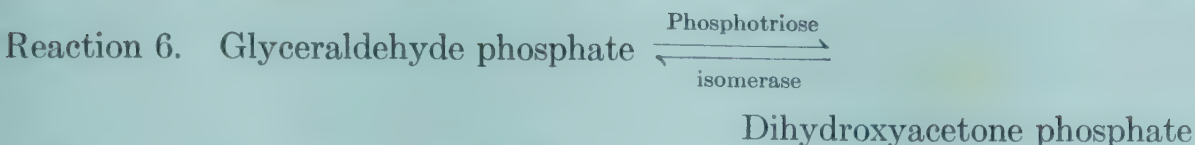
Those enzymes which catalyze the transfer of a phosphate group from ATP are known collectively as *kinases*. It should be noted that in the phosphate ester which forms, the oxygen-phosphorus bond is not energy rich, and so this process consists essentially in the squandering of 12,000 cal. to produce a 3000-calorie linkage. As a result of this great energy difference, this reaction, unlike the previous ones in the sequence, is not directly reversible.

Since it has been necessary at this point to sacrifice a high energy bond, the present score in terms of ATP synthesis is minus 1.

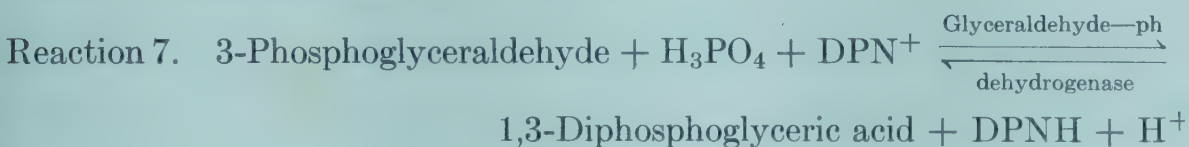


Aldolase was first discovered in yeast, but has more recently been crystallized from rabbit muscle. The mechanism of this reaction, in which the carbon chain is split to yield two triose phosphates, is not known. It is not an hydrolysis, but entails migration of two hydrogen atoms and will probably prove to be far more complex than the simple change indicated in the equation.



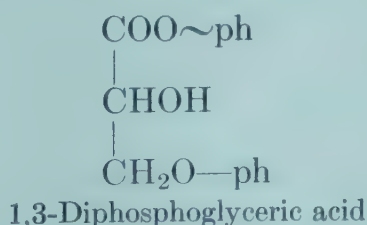


As the triosephosphates form, the attainment of equilibrium is catalyzed by the presence of a specific *isomerase*. As the chart indicates, it is only the glyceraldehyde phosphate which undergoes further reaction in the glycolytic sequence. Consequently if the two triose phosphates were not interconvertible, half of every original glucose molecule would be lost at this point. As a matter of fact the equilibrium between the two three-carbon compounds is very much in favor of the useless ketose phosphate. But in the presence of the isomerase this substance is transformed into the useful aldose derivative as the latter is metabolized, until essentially all the carbon finds its way to the end of the sequence. To indicate this, the latter half of the chart has been written as if two molecules of glyceraldehyde phosphate reacted at this point.



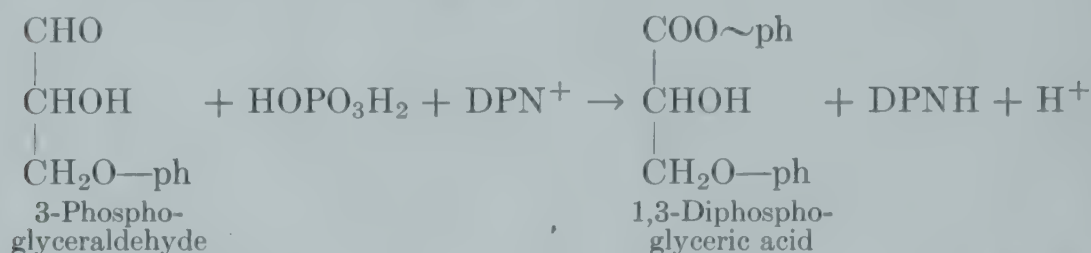
Here for the first time the glycolytic sequence is involved in an oxidation. It will be recalled that oxidizing enzymes consist of a protein and a prosthetic group or coenzyme. Such a coenzyme is the substance indicated by DPN^+ in the equation for Reaction 7. Like the FAD which was previously used as an example of this type of molecule, diphosphopyridine nucleotide (DPN) is capable of reversible oxidation and reduction. The oxidized form is written DPN^+ to indicate that the nitrogen carries a positive charge. When it is reduced it acquires from the substrate an electron and a hydrogen atom, becoming DPNH , while the second hydrogen ion remains in the vicinity, and is later called upon when the DPNH is reoxidized. The complete formulas for DPN^+ and DPNH are given in Chapter 14.

This seventh step in the glycolytic sequence illustrates a common metabolic expedient, the coupling of an exergonic (energy yielding) reaction with one which is endergonic (energy using). The product is 1,3-diphosphoglyceric acid, in which the newly introduced phosphate residue at carbon 1 is held in an energy rich bond. There are two possible ways in which

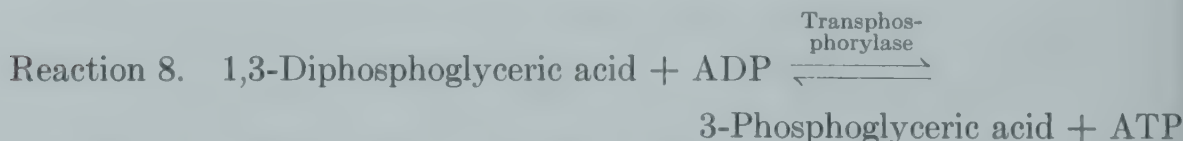


a cell can bring about such a synthesis. If there is available an energy rich phosphate compound such as ATP, and an appropriate enzyme, both the

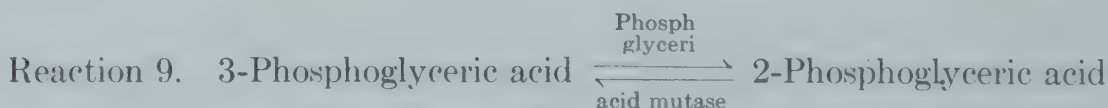
phosphate group and its energy may be transferred to a phosphate acceptor. It is such a transfer which takes place between creatine phosphate and ADP. Failing such a source of energy, synthesis can take place only if it is coupled with a reaction which frees enough energy to form the bond. Reaction 7 of the sequence is of this latter type. The oxidation (dehydrogenation) of glyceraldehyde phosphate yields the energy for synthesis of the new energy rich bond at carbon 1.



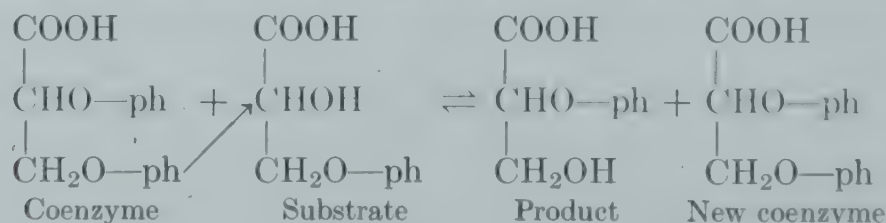
In the earlier discussion of oxidizing enzymes it was noted that the prosthetic group acts as a sort of ferry, being alternately reduced by the substrate and then oxidized in some way, so that one molecule ultimately accepts many pairs of hydrogen atoms from the substrate. In this seventh step of the glycolytic sequence the reoxidation of the DPNH is not immediate, but is brought about in a subsequent reaction of the sequence. At this point then the cell has achieved two newly synthesized energy rich phosphate bonds in the two molecules of diphosphoglyceric acid, and has on its hands two coenzyme molecules in the reduced form.

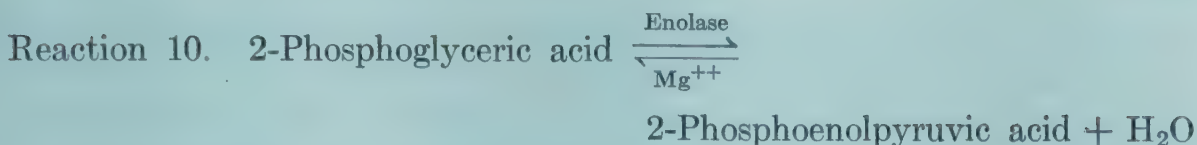


Having now two high energy bonds available, the cell is able to transfer them to ADP and thus to achieve the synthesis of the first two molecules of ATP. It thereby ceases to be a debtor, so to speak, pays back the ATP it "borrowed" in Reaction 4, and has one new molecule of ATP to its credit.

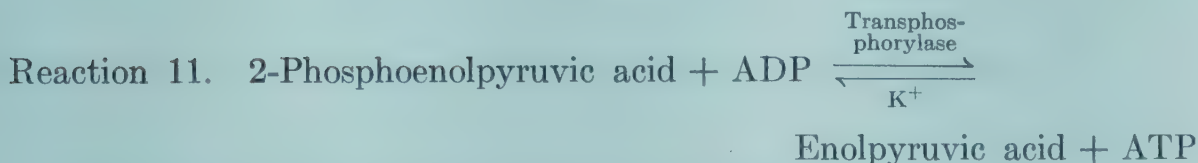
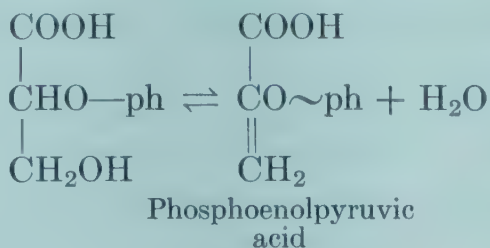


This reaction is exactly analogous to Reaction 2, and the "shift" in the position of the phosphate group is brought about in the same way. The coenzyme is therefore 2,3-diphosphoglyceric acid, and the substrate molecules all go through a stage in which they act as coenzyme for others.

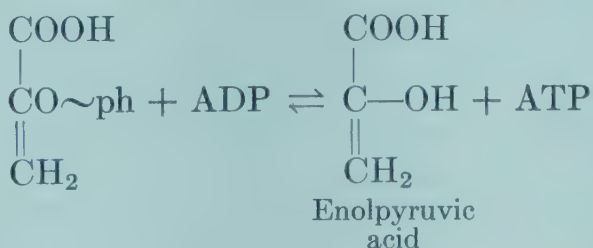




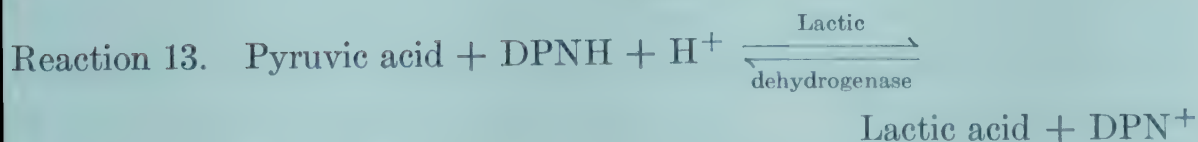
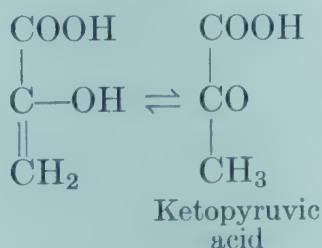
In the removal of water from 2-phosphoglyceric acid, such an intramolecular redistribution of energy takes place that energy is concentrated around the central carbon. Thus again in the sequence a pair of energy rich bonds is generated. The enzyme *enolase* achieves maximum activity only in the presence of magnesium ion.



Again the generation of an energy rich bond is followed by its transfer to ADP, thus adding two more molecules of ATP to the one already formed. The *transphosphorylase* which catalyzes this transfer requires the presence of potassium ion.



This reaction probably takes place spontaneously.



The *lactic dehydrogenase* which catalyzes this reversible reaction was discovered many years ago in experiments in which lactic acid as substrate

was oxidized to pyruvic acid. It is one of several enzymes for which DPN⁺ acts as coenzyme. Hence at this point, under anaerobic conditions pyruvic acid is able to reoxidize the reduced coenzyme previously formed in Reaction 7. Thus lactic acid is formed and the coenzyme is again ready to assist in the oxidation of glyceraldehyde phosphate.

Summary of Glycolysis. Degradation of each six-carbon unit to form two molecules of lactic acid results in a net synthesis of three terminal phosphate bonds in ATP. Assuming for each bond an energy content of approximately 12,000 cal. per mole, this means 36,000 calories made available for muscular contraction by the fermentation of one mole of glucose-1-phosphate. Calculations similar to those by which we estimated the energy change in alcoholic fermentation, show that the loss of free energy involved in the lactic acid fermentation is approximately 57,000 cal. per mole of glucose phosphate. Thus it appears that the muscle manages to divert to its own uses over 50 per cent of the energy made available in glycolysis.

The anaerobic chemical events in muscle may now be outlined as follows:

Preformed ATP initiates the contraction and is itself transformed to ADP. At first this ADP is rephosphorylated at the expense of phosphocreatine, which is present in striated muscle in higher concentration than is ATP. During activity the glycolytic sequence proceeds at such a rate that the concentration of ATP does not change.

Following activity, glycolysis continues until enough high energy bonds have been generated to phosphorylate fully both the ADP and the creatine supplies of the muscle. Thus these compounds at the end of the recovery period are again ready to deal with the next stimulation.

The second high energy bond of ATP is presumably used only under unusual conditions. A special enzyme, *myokinase*, catalyzes the removal of the terminal phosphate residue from ADP, giving rise to the mononucleotide, adenylic acid. As this substance is promptly deaminated in muscle and so destroyed as a carrier of energy rich bonds, this is a wasteful procedure.

Relation of Glycolysis to Oxidation. In times of stress and violent activity the muscles may have to metabolize so fast that they are virtually contracting under anaerobic conditions. But normally they are well supplied with oxygen, both through the agency of the circulating blood, and through their possession of a special oxygen store in oxymyoglobin, or muscle hemoglobin, present in all red muscle. It will be remembered that Fletcher and Hopkins found that excised muscle forms much less lactic acid when it contracts in oxygen than when it contracts in an atmosphere of nitrogen. Furthermore, minced muscle respire rapidly, giving off carbon dioxide and forming little or no lactic acid. These facts all point to the conclusion

that muscle metabolism under normal conditions is aerobic and does not lead to lactic acid formation. It is now believed that the immediate oxidative substrate of muscle, and indeed of nearly all tissues, is not the ubiquitous glucose, but pyruvic acid formed from glucose essentially as outlined in the preceding paragraphs. Under aerobic conditions the hydrogen transferred from glyceraldehyde phosphate to the coenzyme (Reaction 7) is ultimately accepted by oxygen, with formation of water. In this way the supply of oxidized coenzyme is assured. But since these hydrogens are not available to reduce pyruvic acid, lactic acid is not formed. Under these circumstances the pyruvic acid instead of being reduced is oxidized by a mechanism to be considered in the following chapter. It is probable that in moderate exercise the muscles are able to oxidize pyruvic acid as fast as it is formed by the glycolytic sequence, thus obtaining per mole of glucose phosphate metabolized the high energy yield associated with complete combustion. On the other hand, blood lactic acid does rise quite spectacularly during violent exercise. It is therefore probable that under those circumstances part of the pyruvic acid is requisitioned to help re-oxidize the rapidly increasing amounts of reduced DPNH. This would give rise to an equivalent amount of lactic acid, thus completing the anaerobic sequence for some fraction of the carbohydrate metabolized.

Carbohydrates in the Animal Body

The pioneer work in the field of carbohydrate metabolism was done by the great French physiologist Claude Bernard in the middle years of the nineteenth century. When his work began about 1840, and indeed for many years thereafter, it was generally believed that only plant cells could synthesize carbohydrates and that the metabolic activities of animal cells were confined to breaking them down. Nothing was known of the multifarious activities of the liver except that it secreted bile, and the presence of glucose in the circulating blood had not yet been discovered because the methods for the identification of reducing sugars were so insensitive. Many theories had been advanced to account for the clinical entity known as *diabetes mellitus*, in which an excessive volume of urine, *polyuria*, was accompanied by a greater or lesser urinary excretion of sugar, or *glucosuria*. One such theory attributed the disease to a malfunctioning of the digestive process which caused all foods to be transformed in the intestine to sugar.

Claude Bernard set out to discover the metabolic fate of ingested food-stuffs, a subject which interested him as a physiologist and as a clinician concerned with the prevention or cure of diabetes. He began with a study of carbohydrates in the body and in the course of a few years laid the foundation on which has been reared the whole modern edifice of carbohydrate biochemistry. Indeed it is interesting to note, in reading the biochemical literature of the early twentieth century, how many papers

begin, "As Claude Bernard noted in 185—," or "As Claude Bernard prophesied in his paper on ———."

The facts which were established by Bernard's work may be summarized as follows: In the normal animal glucose is continuously formed in the liver, and leaves that gland in the blood of the hepatic vein. This process goes on when an animal is nourished exclusively on protein and also during starvation, showing that the liver is able to synthesize carbohydrate from food protein, or even from the animal's own tissues. The sugar content of normal blood varies between fairly narrow limits, rising during digestion of a meal and falling to its fasting value after about an hour. When for any reason the sugar concentration of the blood rises above a certain value, now known as the *renal threshold*, the excess is excreted in the urine. When certain nerves are injured or cut a glucosuria results, thus proving at least a measure of nervous control of the flow of sugar from the liver. Incidentally this observation led Bernard to the first method for inducing an experimental condition which resembles diabetes. The procedure, known as *piqûre*, consists in puncturing a specific spot in the medulla oblongata, thus causing a rise in blood glucose, or *hyperglycemia*, which in turn leads to glucosuria.

It was Claude Bernard who first used the expressions "external secretion" and "internal secretion" to differentiate the bile, which leaves the liver by way of a duct, from the glucose which he said was "secreted" and which passes directly into the blood and is carried by it to all parts of the body. Bernard expected to find in the liver itself appreciable stores of glucose, waiting to pass into the blood, but found to his surprise almost none. Instead he discovered and named the polysaccharide glycogen, or "glucose-former," which constitutes the body's chief reserve supply of carbohydrate. By confining rabbits to a diet of fat or of meat he proved that liver glycogen may be formed from dietary protein, but not readily from fats.

Thus by the time of Bernard's death in 1878 all the main outlines had been sketched in and Bernard himself had clearly recognized the lability of the whole system. As early as 1855 he had written: "In the liver, stationed like a living laboratory between the intestinal canal and the general circulatory fluid, [the foods] undergo profound changes which serve to maintain the particular equilibrium needed to ensure to the blood of all animals that similarity and constancy of composition which is essential to them."

CARBOHYDRATE EQUILIBRIUM

It is now known that the delicate balance of that "particular equilibrium" is largely maintained through a system of hormonal controls. Internal secretions with antagonistic functions complement or neutralize each other in such a way that blood sugar concentrations are held within narrow

limits even when the food supply varies widely both in quality and in quantity.

The Pancreas and Carbohydrate Metabolism. The first indication that another gland than the liver is concerned with carbohydrate metabolism came in 1893 when von Mering and Minkowski in Germany discovered that extirpation of the pancreas in experimental animals gives rise to a typical case of diabetes. This result can be averted by implantation of an isolated bit of pancreas under the skin, thus showing that the active principle of the pancreas must be an internal secretion. As long as the pancreatic transplant survives, its secretion is absorbed into the blood and carried to the tissues as usual. The action of the pancreatic tissue was later traced to certain special cells known as the islets of Langerhans. It had been noted that the pancreas of patients who had died of diabetes often showed lesions in these cells when the rest of the gland was apparently normal. The first attempts to extract an active principle from the pancreas met with failure, since the hormone itself is a protein and is formed in an organ which also elaborates protein-splitting enzymes. When these substances were extracted together the hormone was destroyed by digestion before the extract could be tested. Finally in 1922 Banting and Best⁵ in Toronto succeeded in bringing about in experimental animals a preliminary atrophy of most of the pancreatic tissue except the islets. They were then able to extract the active hormone, injection of which into diabetic animals caused an immediate remission of symptoms. The active substance was named insulin. It is a protein of low molecular weight (about 12,000) and has been obtained in the form of crystals which always contain a small amount of zinc.

In spite of the spectacular clinical success of insulin, there is still doubt about the exact point at which it exerts its action. In many types of diabetes it certainly brings about a fall of blood sugar and consequently a lowered glucose excretion. But whether this is because it accelerates the oxidation of glucose by the tissues or inhibits its formation from glycogen or promotes its removal from the blood to form glycogen in the liver and muscles is still a matter for argument.

The Anterior Pituitary and Carbohydrate Metabolism. Meantime it has become clear that insulin is only one of several glandular secretions involved in maintaining carbohydrate balance. At the base of the brain lies the

⁵ Frederick Grant Banting (1891-1941) was a Canadian physician who gave up a medical practice to go to J. J. R. MacLeod's laboratory at the University of Toronto to test out his idea for the isolation of insulin. His collaborator in the successful experiments which followed was Charles Herbert Best (1899-) who is now Professor of Physiology at Toronto and Director of the Banting and Best Department of Medical Research. In 1923 the Nobel Prize in Medicine was awarded jointly to Banting and MacLeod who then shared it with their colleagues and collaborators, Best and J. B. Collip. It is one of the special tragedies of the war that Banting's career was cut short by his death in an airplane mishap while on war duty.

tiny pituitary gland or hypophysis which consists of an anterior and a posterior lobe separated by the slender *pars intermedia* (Fig. 13.2). In the anterior pituitary (or *pars distalis*) is secreted a group of so-called "trophic" hormones, the presence of which in the blood exerts the stimulating effect on which depends the development and functioning of the gonads, the thyroid, and the adrenal cortex. Thus the anterior pituitary acts primarily to control and integrate a large part of the endocrine system. Since both the thyroid and the adrenal cortex participate in the regulation of carbohydrate metabolism, the anterior pituitary is indirectly involved through its thyreotrophic and adrenotrophic hormones.

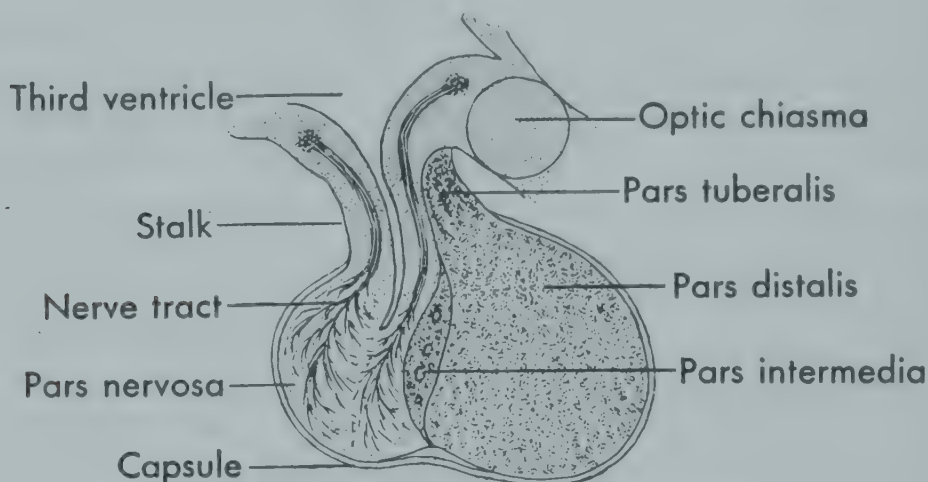


Figure 13.2. Cross section of the pituitary gland.

The suggestion has been made from time to time that the anterior pituitary also exerts a direct influence on carbohydrate metabolism. It was at one time supposed to elaborate a specific "diabetogenic" factor, which acted to oppose the effects of insulin. Since this one small organ secretes so many very potent substances, it has not yet been possible to obtain from it the pure crystalline hormones. This makes it necessary to depend upon such experimental results as may be obtained by extirpation of the gland (hypophysectomy) followed by injection of extracts of varying degrees of purity. In this way it has been shown that the anterior pituitary secretes a substance which controls normal growth. The *growth hormone* has been much concentrated and freed of many of the other active factors and seems to be a definite entity. A second hormone which is believed to exert a direct systemic effect is the *lactogenic hormone* which stimulates the mammary gland to secretion. These two, with the two gonadotrophic, the thyreotrophic, and the adrenotrophic hormones are the six for which there is definite experimental evidence.

As far as carbohydrate metabolism is concerned, the fact is that hypophysectomy results in a profound disturbance of the carbohydrate equilibrium. Absorption of glucose is retarded, the carbohydrate supplies in muscles and liver are rapidly depleted and the experimental animal de-

velops a greatly increased sensitivity to insulin. This interrelationship between the pancreas and the pituitary was dramatically confirmed by Houssay in Buenos Aires. He discovered that removal of the pituitary gland from totally depancreatized animals is followed by amelioration of the diabetic symptoms, and a definite prolongation of life.

But whether the effects just noted result from the removal of some factor specifically concerned with carbohydrate metabolism is still an open question. The way in which the growth hormone controls cell division is not known. It may prove that its primary effect is on some phase of carbohydrate metabolism, for the purified growth hormone does induce just those changes previously ascribed to a separate carbohydrate-regulating factor. In summary then, we know that the anterior pituitary influences carbohydrate metabolism, but whether that influence is direct or indirect or both remains to be discovered.

The Adrenal Glands and Carbohydrate Metabolism. The pair of tiny glands which cap the kidneys in mammals and in most other vertebrates are the

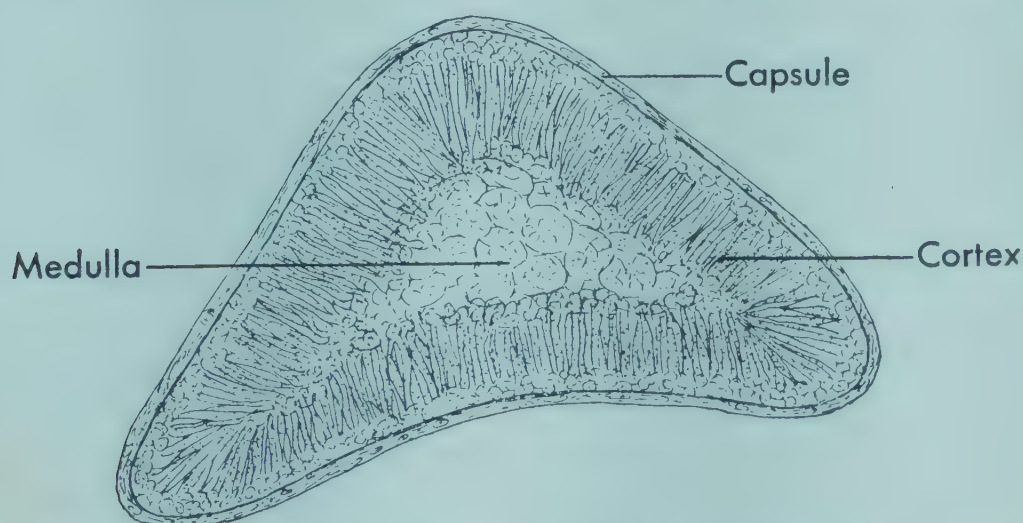
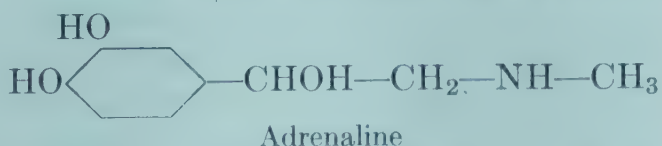


Figure 13.3. Cross section of the adrenal gland, showing the relation of cortex to medulla.

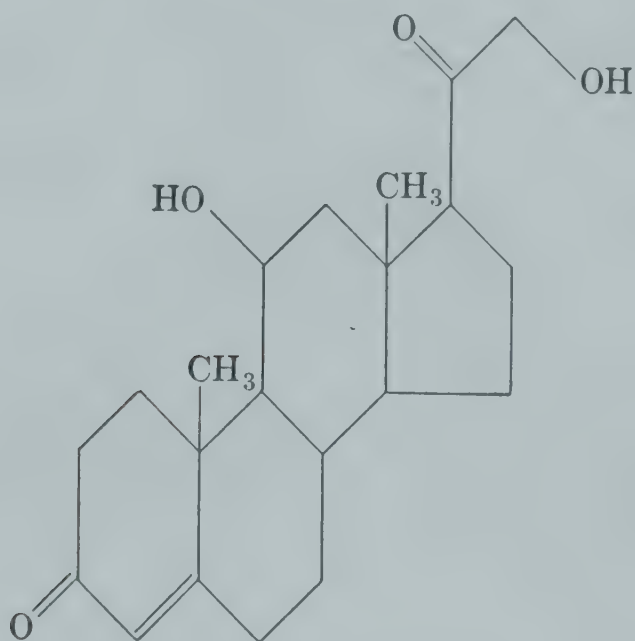
adrenal glands, also known as the suprarenal or epinephral glands. Structurally they consist of two quite distinct parts, the *medulla* or core and the enveloping *cortex* (Fig. 13.3). The adrenaline (epinephrine) which originates in the medulla was the first of the hormones to be isolated in pure form. As the formula shows, it is essentially a substituted amine and



like many amines it exerts a profound pharmacological effect. This includes stimulation of glycogen breakdown both in liver and in muscle. Liver glycogen becomes blood glucose, while the glycogen of the muscles is transformed, as usual, into lactic acid or pyruvic acid.

Whether or not there is a small continuous secretion of adrenaline is not known, but certainly many kinds of sudden stress are met by an immediate secretion of this hormone. Thus when sudden fright calls for rapid use of carbohydrates to supply energy for running away or for giving battle, the adrenaline concentration in the blood rises rapidly and this results in prompt mobilization and utilization of reserve carbohydrate supplies. Since adrenaline is readily oxidized by the tissues its stimulation is of limited duration.

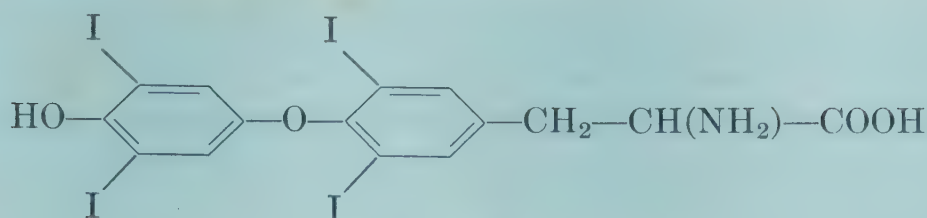
Aside from the function performed by the medulla in emergencies, the adrenals also participate in carbohydrate metabolism through the secretions of the cortex. Extraction of the adrenal cortex yields a solution once known as "cortin." This consists of a complex mixture of steroids of varying potency, all related structurally to corticosterone. Experiments with



Corticosterone

adrenalectomized animals indicate that these steroids act to limit the utilization of carbohydrates and perhaps to promote formation of liver glycogen.

The Thyroid and Carbohydrate Metabolism. Although the active principle of the thyroid gland, thyroxin, was isolated in 1915, it was not until 1926 that its structure was finally proved. By degradation and by synthesis C. R. Harington showed it to be an iodine-substituted aromatic α -amino acid. It is obtained from the tissues as a racemic mixture, but the l-form proves to be the more potent. In the higher mammals absence of the hormone, whether as a result of surgery or disease or of lack of iodine, causes a general slowing of the oxidative rate with which are associated clinical symptoms of more or less severity. Administration of thyroid extract is



Thyroxin

followed by an increase in the basal metabolic rate which manifests itself in an increased oxygen uptake and an increased utilization of all the food-stuffs. Specifically it leads to a reduction of liver glycogen which means that it induces mobilization of glucose to support the increased oxidation.

Results of Hormonal Control. It is clear from this brief discussion that the equilibrium which is established among the carbohydrates in the cells and fluids of the body is a very complex affair. The monosaccharides which enter the portal system during digestion give rise to a temporary increase in the sugar concentration of the blood above its normal resting value. In man this resting value is about 80 mg. per cent, but it varies somewhat from species to species. In the course of about an hour this concentration returns to normal, a large part of the excess being stored in the liver as glycogen. Of the remainder, part is taken up by the muscles and stored as muscle glycogen while the rest finds its way into the cells where it is oxidized. Between meals the glycogen of the liver is called upon to supply a constant stream of glucose which is secreted into the blood. Thus removal of glucose from the blood by the cells is just balanced by *glycogenolysis*, or breakdown of glycogen to glucose. Muscle glycogen does not constitute a general reserve, since even in starvation the store in the muscles is never reduced below a certain minimum value. Evidently at this point the needs of the muscles themselves take precedence over any demands of the organism for fuel.

The hormones which mediate the changes in this complicated system act at a number of points and in a variety of ways. For example the blood sugar concentration may be lowered by an increase in the rate of oxidation in the cells, provided there is no corresponding increase in the rate of secretion of glucose into the blood by the liver. Or it may be raised either by inhibition of *glycogenesis*, thus retarding removal of glucose to form glycogen, or by stimulation of glycogenolysis. At present there is much uncertainty about the specific effects of the individual hormones, but in Figure 13.4 an attempt has been made to outline the various changes undergone by the carbohydrates in normal animals and to indicate the controlling hormonal influences. The chart is applicable in its entirety only to the higher mammals, but presumably a similar system of checks and controls is operative in all animals which are high enough in the scale to have an endocrine system. The result is that the blood of an animal contains a nearly constant supply of glucose at all times except

during digestion. This sugar is carried in the capillaries to all the cells of the body where it serves as one of their main respiratory substrates. The energy derived from its oxidation is used by the cell for any operation in which work must be done, such as synthesis, secretion against an osmotic gradient, and muscular contraction.

THE CARBOHYDRATE EQUILIBRIUM

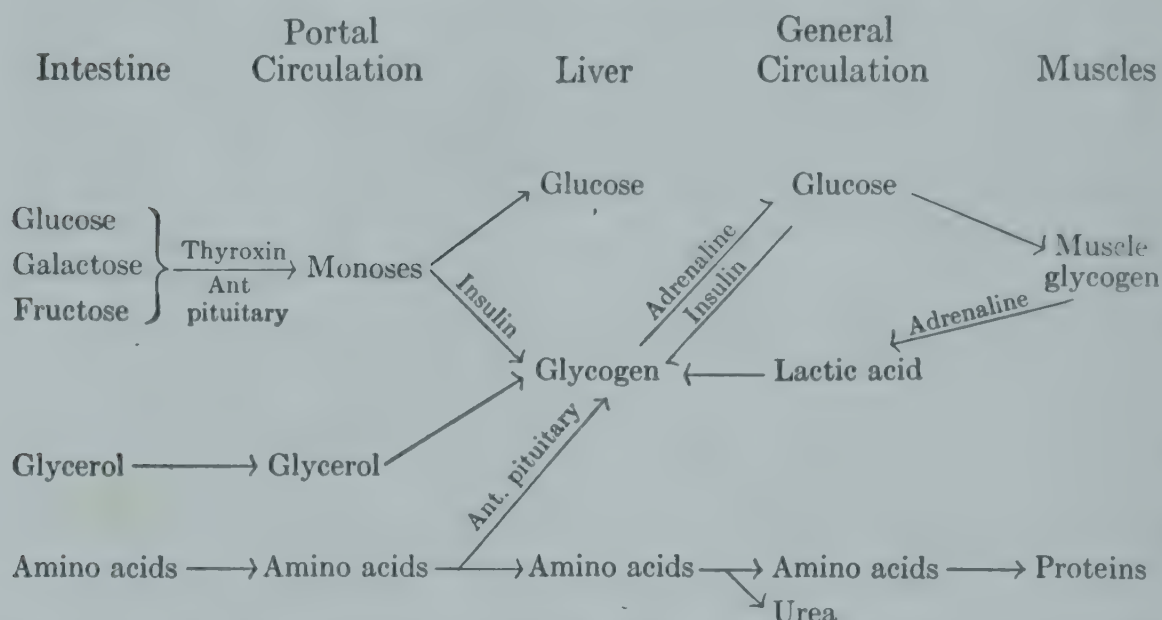


Figure 13.4. The Carbohydrate Equilibrium. The parts played by the various hormones in maintaining the carbohydrate equilibrium in the body.

Glycogen Precursors. Although the major source of liver glycogen is the carbohydrate part of the diet, various other compounds have also proved to be glycogen formers. When noncarbohydrate compounds give rise to glycogen the process is known as *glyconeogenesis*. From the proteins, only about half of the amino acids give rise to glycogen and, as would be expected, few of the essential acids are included on the list (see Chapter 11). Fatty acids have recently been shown to be glycogen precursors, as is also the glycerol part of the fat molecule. So likewise are various other three-carbon compounds which arise in the course of metabolism, notably lactic acid and pyruvic acid. As was noted in connection with β -oxidation, fatty acids with an odd number of carbon atoms, such as propionic acid and valeric acid, add to the glycogen stores more readily than do those with an even number. The latter are more likely to lead to acetoacetate formation, since they can be split completely into two-carbon units.

Carbohydrates in Plants

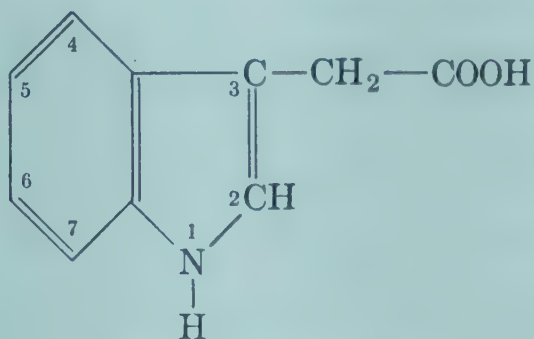
It is now generally accepted that the main respiratory substrate of plant cells as of animal cells is glucose. In the green leaves of a typical higher plant, glucose and other hexoses are synthesized during daylight hours

and may be translocated through the phloem to all parts of the plant. Carbohydrates synthesized in excess of immediate needs are stored, chiefly as starch or other polysaccharide in some plants, occasionally as sucrose in others. This stored material, which provides nourishment during the hours of darkness, is degraded to glucose or fructose when the cells require it. Perennials store in their roots the starch which will be needed by the young shoots when growth is resumed in the spring. In many seeds the reserve is stored chiefly in the form of fat. In such seeds the transformation of fat to carbohydrate can be demonstrated by comparing the composition of seeds with their seedlings before the formation of chlorophyll makes photosynthesis possible. For example, in sunflower seeds, fat may make up over 55 per cent of the total dry weight but this drops to less than 25 per cent in the seedling. Part of this loss undoubtedly comes from oxidation of fatty acids, since the R.Q. is approximately 0.7, but about one third of the lost fat appears in the seedling as newly formed sugars and polysaccharides.

There must then be in plants an equilibrium quite as complex and as delicately balanced as the one which obtains in the higher vertebrates. Since much less is known of the agencies which control its fluctuations, we merely note here that there is in the plant such an harmonious interplay of factors, including enzymes, that the living cells are provided with a continuous supply of hexose, usually glucose.

PLANT HORMONES

Although there are no plant glands which corresponds to the animal endocrine system, the plant does control its economy by chemical means which are apparently analogous. In such active centers as the tip of a growing shoot there is elaborated a growth stimulant or *auxin*, and if this tip is removed, normal extensional growth is interrupted. Several pure substances have been shown to stimulate plant growth. Among these the one which has actually been obtained from plant tissues is indole-3-acetic acid. Related to the latter is indole-3-butyric acid which stimulates root formation wherever it is applied, so that a tomato plant brushed with it from the ground level to the tip of a leaf will grow a white fringe of roots



Indole-3-acetic acid

all along the stem and out onto the leaf! The root-forming hormones are used to facilitate the rooting of cuttings, while the growth hormones if sprayed on weeds in sufficient concentration cause such rapid and abnormal growth that the plants finally die. Undoubtedly the field of plant hormones should be a fertile one in the next few years, but at the moment there is nothing known of the mechanism of their actions.

Carbohydrate Metabolism in Cells Other Than Those of Muscle

ALCOHOLIC FERMENTATION

Chemical studies of alcoholic fermentation began in 1897 with the classical discovery of Eduard Buchner that cell-free yeast extracts were able to bring about fermentation of sugar. During the next twenty years a long series of papers on fermentation came from Buchner's laboratory, overlapping in time the studies on the same subject by Arthur Harden and his collaborators in London and by Carl Neuberg in Berlin. These three carried out or directed much of the most important early work in the field. The basic reaction may be simplified to



As the chemical changes in fermentation were slowly elucidated, it began to appear that they involved many of the same substances which take part in the chemical events in muscle. Ultimately it was proved that step for step, in nearly every detail, the chemical reactions leading to ethyl alcohol are identical with those by which lactic acid is formed. At only two points do the two sequences diverge.

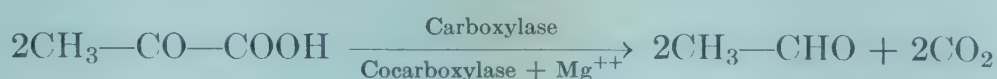
If the yeast is acting upon starch, the initial stage is a phosphorylation of the polysaccharide which yields glucose-1-phosphate exactly as in the analogous breakdown of glycogen. From glucose-1-phosphate, the 6-ester is formed as in muscle. But yeast, like many types of animal cells, can also use glucose as substrate and under these conditions the alcoholic sequence is initiated by a reaction which has no counterpart in muscle metabolism. With glucose as substrate the first step, catalyzed by the enzyme *hexokinase*, brings about transfer of a phosphate group from ATP directly to carbon 6 of glucose.



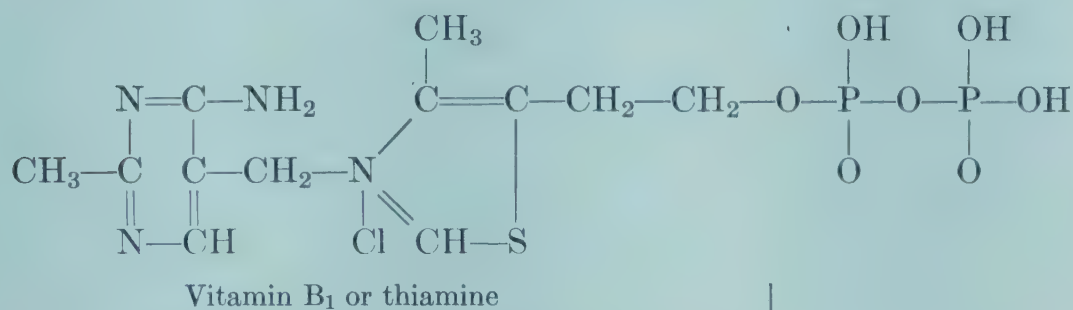
Here a low energy phosphate bond is formed at the cost of an energy rich bond of ATP. Thus, in terms of triphosphate synthesis, glucose is a less efficient substrate than starch or glycogen.

The intermediate stages between glucose-6-phosphate and pyruvic acid are identical in alcoholic and in lactic acid fermentation. But when pyruvic acid has formed, the two paths of anaerobic metabolism diverge for the

second time. The yeast cell contains an enzyme *carboxylase* which splits out carbon dioxide from pyruvic acid, forming acetaldehyde.



As the equation indicates, carboxylase is active only in the presence of magnesium ions and of a coenzyme known as *cocarboxylase* which is a derivative of vitamin B₁ or thiamine.



Vitamin B₁ or thiamine

Cocarboxylase or diphosphothiamine

The importance of thiamine in animal nutrition is believed to lie in its ability to form this phosphorylated derivative which is essential to other enzymic reactions than this one under discussion.

It will be remembered that under anaerobic conditions the glycolytic sequence gives rise to a reduced coenzyme at the step at which triosephosphate is dehydrogenated. In muscle this reduced coenzyme is later reoxidized by pyruvic acid, which becomes lactic acid in the process. In alcoholic fermentation on the other hand the reoxidation is brought about by reduction of the acetaldehyde which is the product of the decarboxylation of pyruvic acid. In this process the acetaldehyde is itself reduced to ethyl alcohol.

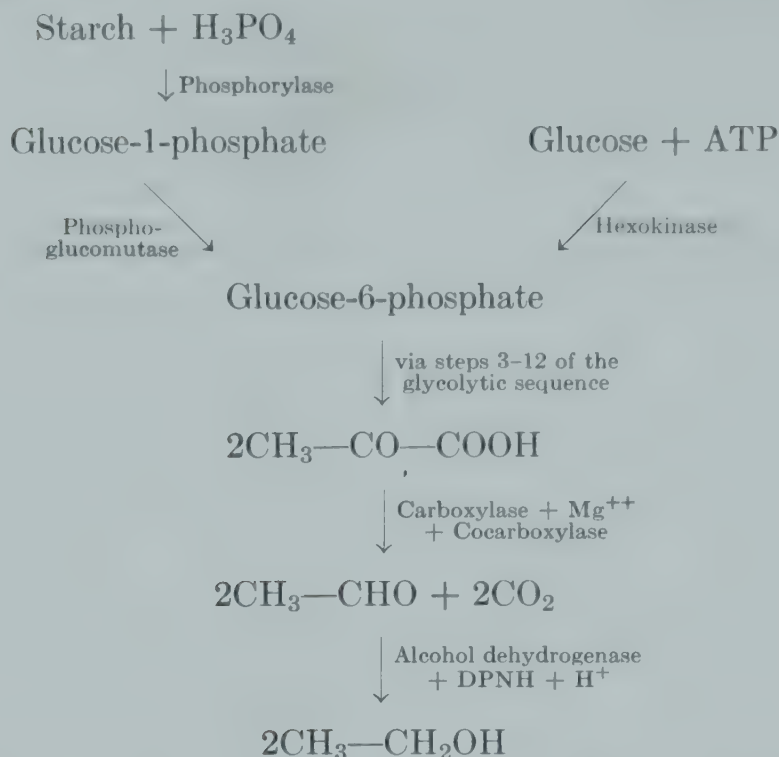


Thus the coenzyme is freed to enter again upon hydrogen transport, and in the last two reactions two moles of alcohol and two of carbon dioxide have been formed from each mole of glucose metabolized. This is in accord with the known quantitative relations in yeast fermentation.

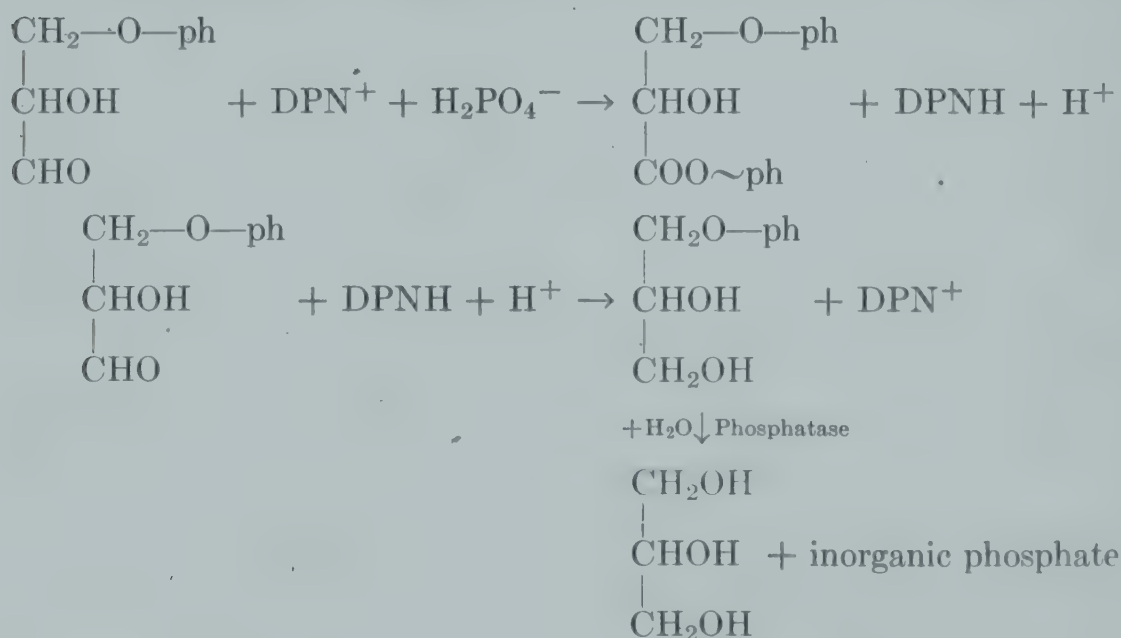
The fact that the enzyme which causes reduction of acetaldehyde is labeled "alcohol dehydrogenase" should cause no difficulty. Like most enzymes it acts reversibly, and it happens to have been discovered first in experiments with cellular oxidation of alcohol.

The scheme below outlines the steps which are characteristic of the main fermentation reactions of yeast.

Reactions of Alcoholic Fermentation



By-Products of Alcoholic Fermentation. Although the reactions just given describe the main events in alcoholic fermentation, it was recognized very early that other products are always formed in small amounts. The chief of these are glycerol and acetic acid with smaller amounts of succinic acid and of various alcohols. By controlling the conditions under which fermentation takes place, it is sometimes possible to increase the yield of one by-product or another. For example, in an alkaline medium the yield of glycerol is greatly increased. This is believed to be due to the fact that



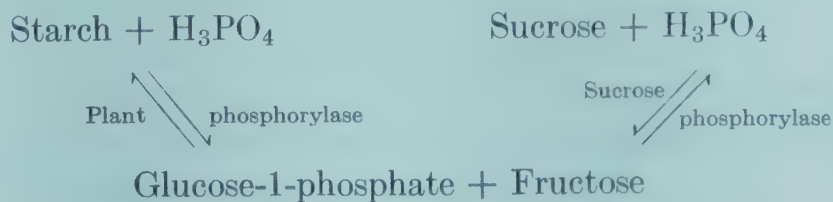
in alkaline solution the labile acetaldehyde undergoes a *dismutation* or Cannizzaro reaction, forming alcohol and acetic acid. This removes from the system part of the substance which normally is reduced in oxidizing the reduced coenzyme. Under those circumstances the reoxidation of the coenzyme is brought about by reaction with part of the triosephosphate, which is thus reduced to phosphoglycerol. Removal of the phosphate group follows through the agency of a *phosphatase*, an enzyme which catalyzes hydrolysis of phosphate bonds. This reaction amounts then to a two-step dismutation or oxidation-reduction of the triosephosphate, one molecule being oxidized by indirect transfer of two hydrogens to another molecule. The first of these two reactions is of course Reaction 7 of the glycolytic sequence.

To account for the formation of all the various by-products of fermentation would take us too far afield. Remembering the reactivity of acetaldehyde and of pyruvic acid, to mention only two substances in the complex reaction mixture, it should be easy to understand that they alone might well give rise to a number of different products. Furthermore, in the living yeast other reactions than those of carbohydrate metabolism are going forward simultaneously. The cell therefore has available also the products of amino acid and of fatty acid catabolism, and these too may interact with each other or with members of the carbohydrate sequence. Thus there are formed a number of substances in trace amounts, including the various alcohols to which are due the special aromas and flavors of different wines.

GLYCOLYTIC SEQUENCE IN PLANTS

It is generally believed that utilization of carbohydrates by plant cells follows the pathways just outlined for animal tissues and for yeasts. There have been attempts to find evidence for direct oxidative attack on hexose molecules and indeed the molds *Aspergillus niger* and *Penicillium notatum* have yielded a *glucose oxidase* which catalyzes the direct oxidation of glucose to gluconic acid. But this reaction is probably of limited importance, with the bulk of the carbohydrate in normal healthy plant cells being metabolized by way of the glycolytic sequence followed by oxidation of the pyruvic acid.

The chief reserve substrates of plant respiration are starch and sucrose, both of which are related as indicated below to glucose-1-phosphate. Plant tissues have yielded both a phosphorylase which brings about phosphorylysis of starch and also a specific sucrose phosphorylase.



Thus from the two common storage carbohydrates a phosphorylated hexose can be formed without the use of any high energy phosphate compound. There is now a good deal of evidence that plants metabolize this glucose-1-phosphate by the familiar glycolytic pathway. Various phosphorylated intermediates have been isolated, and several of the individual enzymatic reactions have been demonstrated in cell free plant extracts. Pyruvate has been identified as a product of plant enzymes acting on phosphoglycerate. These and many similar facts all point to the probability that the higher plants possess the enzymatic machinery to bring about glycolysis of sugars and polysaccharides with formation of pyruvate.

It has long been known that under anaerobic conditions plants can bring about alcoholic fermentation with evolution of carbon dioxide. This presumably results from decarboxylation of part at least of the pyruvic acid, with subsequent reduction of the acetaldehyde. Whether another part of the pyruvate is reduced to lactic acid under these conditions is not definitely known. However, since lactic dehydrogenase is known to be present in many plant cells, it seems likely that they can also reoxidize reduced coenzyme with pyruvic acid, thus forming small amounts of lactic acid.

Carbohydrate Synthesis

Since every reaction of the glycolytic sequence except one is reversible, it is reasonable to assume that synthesis of carbohydrate in living cells takes place by what amounts to reversal of this same series of reactions. As we shall see in Chapter 15, recent experiments with isotopes show that photosynthesis probably follows this pathway, or a very similar one.

The one irreversible reaction in the sequence is Reaction 4, in which a high energy bond is sacrificed to form fructose diphosphate from the monophosphate. Since the exact reversal of this reaction would require synthesis of a new energy rich phosphate bond, it is impossible unless coupled with some new source of energy. But as far as the *carbohydrate reactants* are concerned, the reverse step presents no difficulties. It requires only hydrolysis of the phosphate bond at carbon 1, and the enzyme phosphatase which catalyzes such reactions is found in all cells. This means that *in vivo* the whole series of reactions from starch or glycogen to pyruvic acid is completely reversible as far as the carbohydrate molecules are concerned. Experiments with liver slices and isotopically labeled pyruvic acid indicate that part at least of the isotope is incorporated into glucose by a reversal of glycolysis.

The first laboratory synthesis of polysaccharides became possible with the discovery of the enzyme phosphorylase in animal tissues in 1937 and in potato tubers in 1940. Up to this time it had been assumed that the first step in the utilization of polysaccharides by living cells was an hydrolysis; and attempts to reverse this reaction had met only with failure. But as soon as the purified phosphorylases became available, and were

treated with their natural substrate, glucose-1-phosphate, *in vitro* polysaccharide synthesis became a reality. Phosphorylases have been isolated from yeast, from muscle, liver, heart, and brain and from many different plant tissues. They all act upon glucose-1-phosphate to form some type of polysaccharide, and to set free inorganic phosphate.

Two facts of special interest have emerged in more recent studies of polysaccharide synthesis. The first is that highly purified phosphorylase catalyzes formation of 1-4' linkages only, giving rise to a polysaccharide of the amylose, or unbranched, type. Thus the phosphorylases prepared from muscle and from potato yield similar linear products, though the normal polysaccharide of muscle is the branched glycogen, and starch normally consists largely of the branched amylopectin. This discrepancy is believed to be due to the lack in the purified enzymes of a second "branching factor" required for formation of 1-6' links at the branching points. The Coris have shown that there is such a factor present in brain, heart, and liver phosphorylase preparations, and in Haworth's laboratory a similar factor, referred to as Q enzyme, has been isolated from potato juice. Thus the synthesis of branched polysaccharide requires the collaboration of two separate enzymes, one of which brings about formation of amylose or an amylose-like unbranched polyglucose. Through the agency of the other, glucose units become attached at position 6 to initiate formation of the branches.

The second discovery in this field is that with purified enzymes no synthesis results unless the system is "primed" with a small amount of branched polysaccharide. Either starch or glycogen may be used, but the smaller dextrans are much less effective. The plant and animal phosphorylases act equally well with either plant or animal polysaccharide, and appear to bring about synthesis by a lengthening of existing polysaccharide chains.

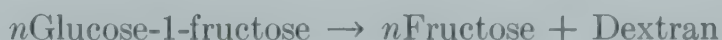
As a result of more recent work with bacterial enzymes, it now appears that use of the phosphate link at carbon 1 to provide energy for formation of a glucosidic link is quite general. Sucrose is synthesized under the influence of a specific sucrose phosphorylase, through the interaction of glucose-1-phosphate and fructose.



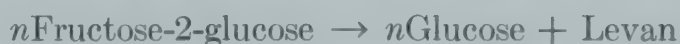
Other disaccharides may be formed also by replacing fructose with sorbose or with one of several pentoses. On the other hand the enzyme seems to be specific for glucose-1-phosphate, for several similar phosphate esters of other sugars did not react.

Obviously our present knowledge of synthetic mechanisms is still elementary, but the plan is beginning to take form. If the fundamental substrate is glucose, it must first be phosphorylated before it can be condensed with other molecules. This is achieved by interaction with ATP

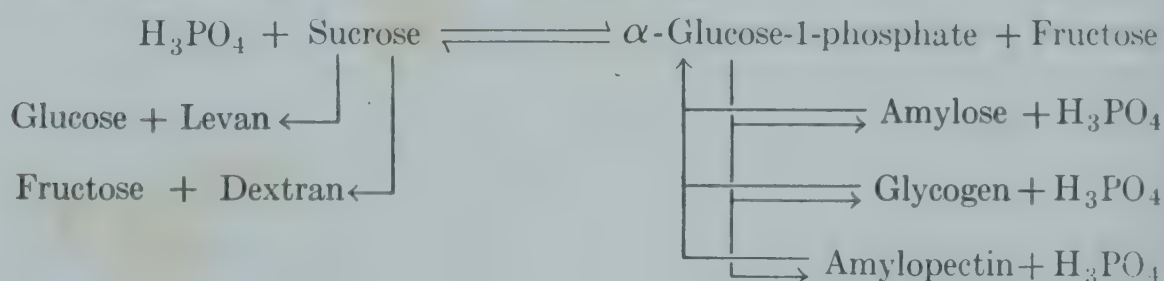
in the presence of hexokinase. Here an energy rich bond is sacrificed, but the small percentage of its energy which is stored in the phosphate link is sufficient to allow later formation of a glucosidic bond. The glucose-1-phosphate may then react with fructose to form sucrose, or with itself to build up polysaccharide molecules. Furthermore, the energy of the glucosidic bond can also be used for synthesis much as the low energy phosphate bond is. For such a purpose glucose-1-fructose (sucrose) is analogous to glucose-1-phosphate, and will react by exchange of one glucosidic bond for another. Thus the synthesis of plant or bacterial dextrans, or glucose polysaccharides, can be represented schematically as follows:



Similarly sucrose may be thought of as fructose-2-glucose, using the energy of the glucosidic link to synthesize a levan, or fructose polysaccharide.



Thus the known syntheses of polysaccharides by plants, bacteria and animals may be represented by the following scheme, taken from a paper by Avineri-Shapiro and Hestrin of the Hebrew University in Jerusalem.



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Study Questions

1. What evidence is there that "fermentation" is a wasteful way of using glucose? Why must this be true? What was Pasteur's definition of fermentation?
2. What is now believed to be the function of the series of reactions which we call the glycolytic sequence? How is it related to muscular contraction? How to the metabolism of glycogen?
3. What was the original idea about the relation of lactic acid to muscular contraction?
4. How did Meyerhof relate the anaerobic and the aerobic phases of muscle metabolism? Why did the idea arise that the oxidative phase was a "clock-winding" process?
5. What was the practical importance of the discovery that an active extract could be made from muscle?
6. What was the first experimental evidence for believing that hexose phosphates play a role in muscle chemistry?
7. What is the formula for creatine phosphate, and what is believed to be its role in muscle chemistry?
8. Indicate some of the methods which were used to determine the individual steps in the glycolytic sequence.
9. Which is the oxidative step in glycolysis? How is the reduced coenzyme normally re-oxidized? How is it re-oxidized under anaerobic conditions?

10. Write the formulas of the substances involved in the formation of the high energy bonds in the course of glycolysis. Write the equation for the reaction in which they are transferred to ADP.
11. What kind of reaction does a "mutase" catalyze? What is the mechanism of this reaction?
12. What type of reaction is catalyzed by a "transphosphorylase"?
13. How does the glycolytic sequence in alcoholic fermentation differ from the muscle sequence?
14. What enzyme has been found capable of synthesizing polysaccharide from a glucose derivative in the laboratory? What type of polysaccharide is formed?
15. What additional enzyme is needed to bring about the synthesis of a branched polysaccharide from glucose-1-phosphate?
16. What is meant by the "priming" of polysaccharide synthesis? How is the substance added to prime the reaction believed to act?
17. How is carbohydrate believed to be formed from pyruvate in living tissue?

Biological Oxidations

In periods of transition, dogmatic treatises are understandably difficult [to write] because a work is obsolete before it is finished and there is the risk of a doctrine's being overthrown before it has been completely formulated.

CLAUDE BERNARD: *Leçons de Physiologie* (1855)

In recent chapters we have considered the various known types of reaction by which the molecules of foodstuffs are degraded. We have seen how amino groups may be transferred from one amino acid to another by way of a keto derivative, and how ammonia may be split out and synthesized into urea or reserved for future use as an amide. We have found that there is evidence for believing that fatty acids are broken down and built up by way of two-carbon units, and have traced the elaborate series of chemical changes by which glucose or glycogen is transformed into pyruvate. But at no point have we found any indication of the way in which molecular oxygen plays its part in metabolism, or considered the actual formation of water or carbon dioxide. It is the purpose of this chapter to bring together what is now known of the mechanisms of these two terminal reactions.

Formation of Water

EARLY THEORIES

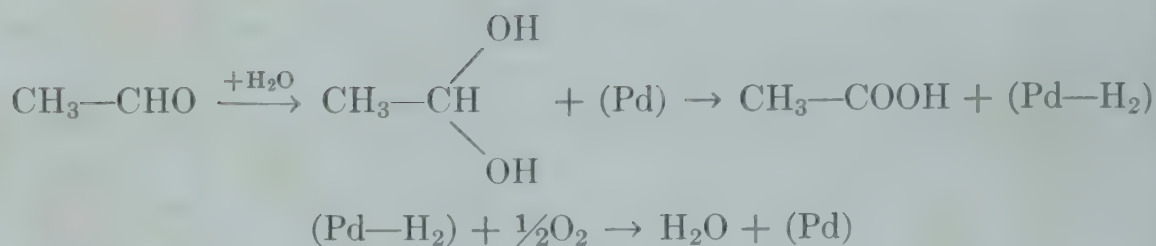
One of the earliest papers on "respiration" is that of Mayow, published in 1668, in which it is assumed that air is used in some way in the lungs. Later writers suggested that oxidation takes place in the blood. Then about the middle of the nineteenth century Hoppe-Seyler discovered the oxygen-transporting function of the blood, and thus showed that the actual oxidations take place in the tissues. Pflüger believed that some sort of intracellular preparation must precede the actual oxidative changes, and postulated incorporation of oxygen into a giant organic molecule which he called "inogen." This was supposed to "explode" when conditions were ripe for oxidation. This first phase in the cycle of oxidation theories ended when it was proved that oxygen is not stored in any form in the tissues.

In the early years of the present century work with enzymes of plants, of animals, and of bacteria led to the formulation of a number of theories designed to explain oxidation in living tissue as a single, specific "activation." Of these theories two have survived and been incorporated in modified form in modern theories of biological oxidation. The first relates biological oxidation to the activation of oxygen; the other postulates an activation of the hydrogen of the metabolites.

Activation of Oxygen. Many of the earliest attempts to explain oxidation assumed a transformation of molecular oxygen into some more active substance such as ozone or atomic oxygen. Beginning about 1920 Warburg elaborated and vigorously defended the rather similar idea that physiological oxidations depend upon a specific catalytic activation of molecular oxygen by iron or an iron compound. His evidence was perforce indirect, but he presented a telling case. He showed that living cells all contain small amounts of iron and that various substances such as hydrogen sulfide and potassium cyanide, known to react with iron or to break up its organic compounds, also inhibit cell respiration. He found that "blood charcoal," that is, the impure carbon made by charring whole blood, catalyzes the oxidation of amino acids, and that its efficacy as a catalyst is roughly proportional to its iron content. Although he did not at first succeed in isolating a "respiratory enzyme" he did carry out a most brilliant study of the properties *in situ* of a substance we now know to be one of a number of enzymes concerned with cellular oxidation (see p. 468). His conclusion was that "the respiratory ferment is the sum of all the catalytically active iron compounds" in the cells, and that the function of this catalysis is the activation of oxygen.

Activation of Hydrogen. Meantime, in 1912 Wieland¹ had suggested, on the basis of a different set of experiments, an entirely different mechanism for the oxidative reactions of living cells. He noted that palladium black as a catalyst brought about the oxidation of moist acetaldehyde to acetic acid even when air was excluded. Under these circumstances some molecular hydrogen was found trapped in the spongy metal. He found further that this hydrogen did not accumulate when oxygen was present, nor in the presence of other easily reducible substances such as benzoquinone or methylene blue. The reaction of acetaldehyde with oxygen was thus formulated as taking place in two steps. In the first "activated hydrogen" is removed from the hydrated aldehyde by the catalyst; in the second this hydrogen is transferred to oxygen with formation of water.

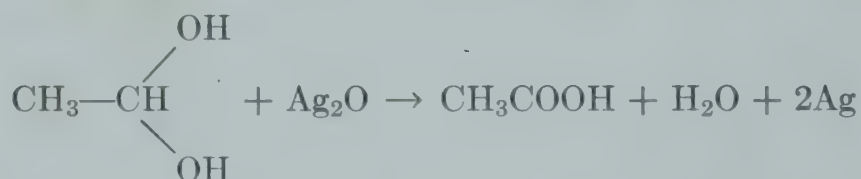
¹ Heinrich Wieland (1877–), Professor at the University of Munich, is an outstanding biochemist whose brilliant work in the first quarter of the twentieth century contributed largely to the rapid growth of our understanding of the mechanism of biological oxidations. He was awarded the Nobel Prize in 1927.



According to Wieland the essential catalysis is the activation of hydrogen of the hydrated substrate, which then functions as a *hydrogen donor*. The palladium acts as a *hydrogen carrier* and may pass on its hydrogen to oxygen or to benzoquinone or to methylene blue. This interpretation served to clarify a great number of different reactions. For example, moist silver oxide readily oxidizes acetaldehyde to acetic acid; but if the oxide is dry and the anhydrous reagents are mixed in dry benzene, no



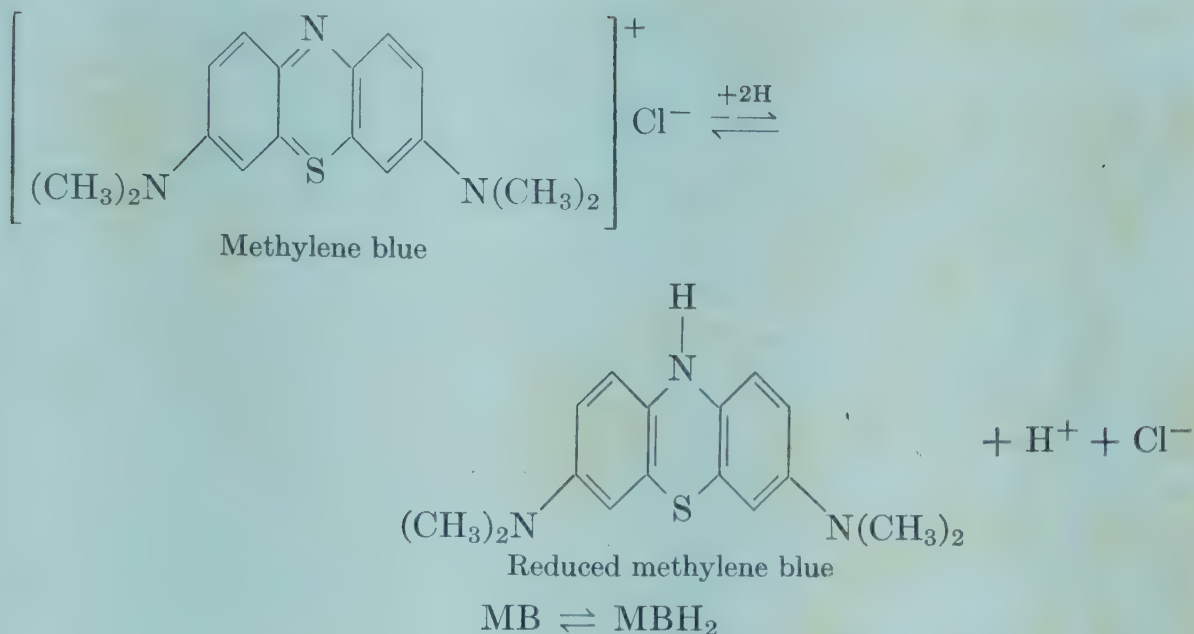
oxidation takes place. In this reaction catalysis is not involved, but again the results are best explained by assuming a preliminary hydration of the aldehyde and a subsequent oxidation which is really a removal of hydrogen. The fact that in many catalytic oxidations the hydrogen can apparently



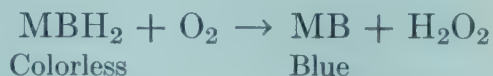
be passed on either to oxygen or to such an artificial hydrogen acceptor as methylene blue led to the view that the role of oxygen is nonspecific and that the cell catalysts act only upon hydrogen of the metabolites. The work of Thunberg (see following section) lent support to Wieland's theory of hydrogen activation, but gradually it became apparent that the truth was to be sought not in a decision between but in a synthesis of the two conflicting theories. It was Szent-Györgyi who first realized that both Warburg and Wieland were right and that to explain cellular oxidations we must assume both an activated hydrogen and an activated oxygen.

THE THUNBERG TECHNIQUE

The significance of Wieland's experiments with methylene blue was greatly increased by the discovery that living tissue contains a great number of catalysts capable of transferring hydrogen from various organic compounds to this dyestuff. Methylene blue is a complex heterocyclic compound with several resonance forms, but the formulas given will serve to indicate its reversible oxidation-reduction. Of the two hydrogens involved, one attaches itself to the dye, the other becomes a hydrogen ion.



The oxidized form of the dye is bright blue, while the reduction product is called a leuco-compound, from the Greek *leukos*, meaning white. In the presence of oxygen the leuco form regains its color. It is said to be *autoxidizable*, since this transformation does not require a catalyst.



When methylene blue is added to any one of a wide variety of minced plant or animal tissues, or to suspensions of yeast or bacteria, the dye is rapidly reduced, except perhaps near the surface where autoxidation keeps it in the oxidized form. Since boiled tissue brings about no such bleaching, the reaction must be enzymic. The group of enzymes which thus bring about the transfer of hydrogen to methylene blue are known as *dehydrogenases*. Although methylene blue is not a normal cell constituent it has been convenient to use the color change which it undergoes to detect the presence of hydrogen-activating enzymes. Obviously it was necessary to reason cautiously from such experiments, but in point of fact they have been justified. Normally in the cell other reducible compounds are present which react very much as methylene blue does to accept hydrogens from oxidizable substrate molecules.

In the hands of the Swedish physiologist Torsten Thunberg the methylene blue technique was developed not only to test for the presence of dehydrogenases in tissue, but also to measure their concentration. The reaction is usually carried out in a "Thunberg tube" (Fig. 14.1) so arranged that it can either be evacuated or be flushed out with nitrogen or other inert gas. With autoxidation of the dye thus prevented, it has been found that the length of time required for the bleaching of a given amount of dye under standard conditions is inversely proportional to the amount of dehydrogenase present.

When the minced tissue is washed repeatedly before being tested, it is found to bleach the dye much more slowly. The rate of reduction can then be greatly increased by adding to the system any one of a large number of organic substances, such as lactate or glucose or alcohol. This



Figure 14.1. A Thunberg vacuum tube. The enzyme source is placed in the hollow stopper and the methylene blue and substrate in the tube. After evacuation of the tube the enzyme is tipped in and the time required for decolorization is measured.

indicates that the enzymes are intact, but that the washing has removed the soluble cell substrates which previously donated hydrogen to the dye. That an enzyme system is involved becomes clear when the substrates are mixed with methylene blue in the absence of tissue, or in the presence of boiled tissue. Under those conditions no bleaching ensues.

By the use of the Thunberg technique it has been proved that plant and animal tissues as well as yeast and bacteria contain a wide variety of dehydrogenases. As might have been expected, the number of substrates which can be dehydrogenated by bacteria is enormous and includes many for which multicellular organisms have no enzymes. This reflects the wide variety of metabolic types found among bacteria. On the other hand there are many dehydrogenases which have been found in every type of tissue investigated. Succinic and lactic dehydrogenases, for example, are widely distributed. So also is the triosephosphate dehydrogenase which catalyzes the single phosphate-coupled oxidation of the fermentation cycle. Thus it seems likely that in most aerobic cells the same dehydrogenases function in exactly the same way to activate the hydrogen of their substrate molecules.

COENZYMES

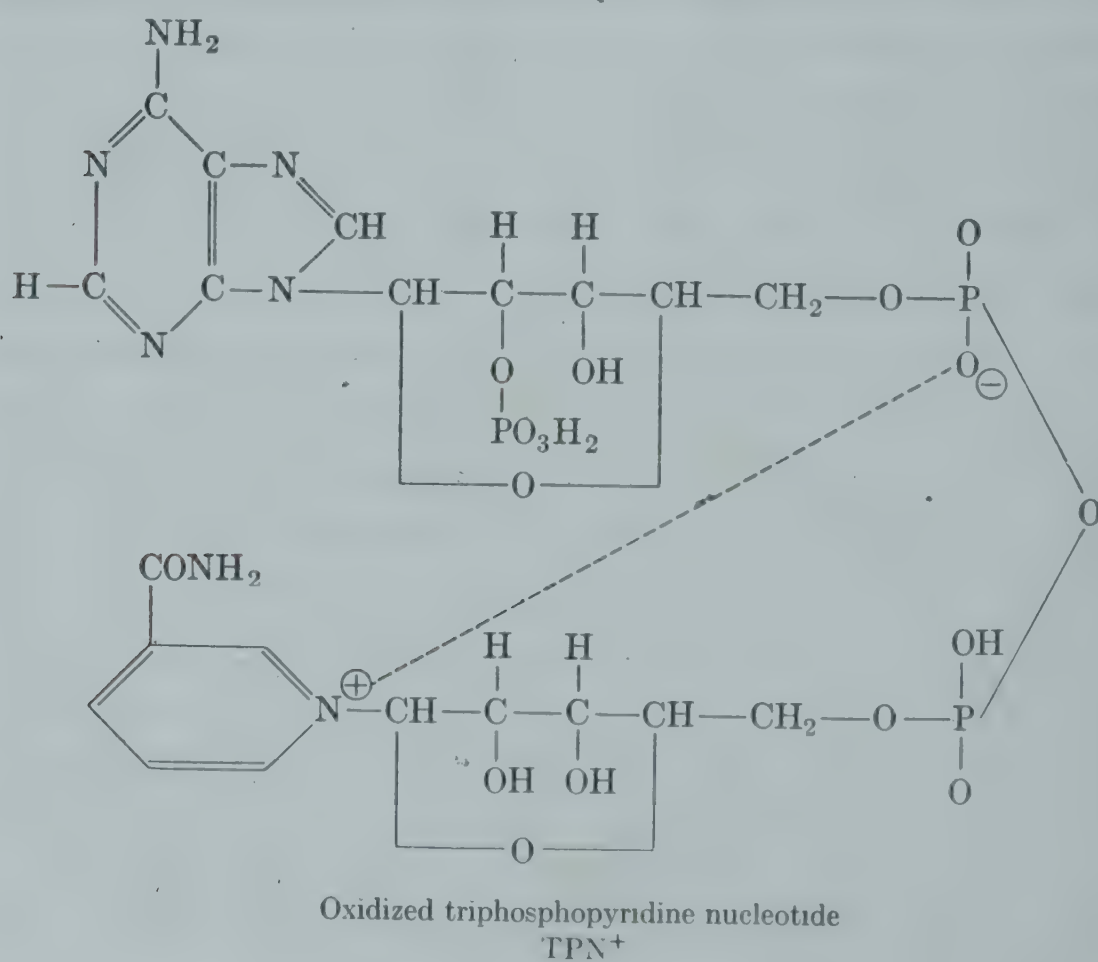
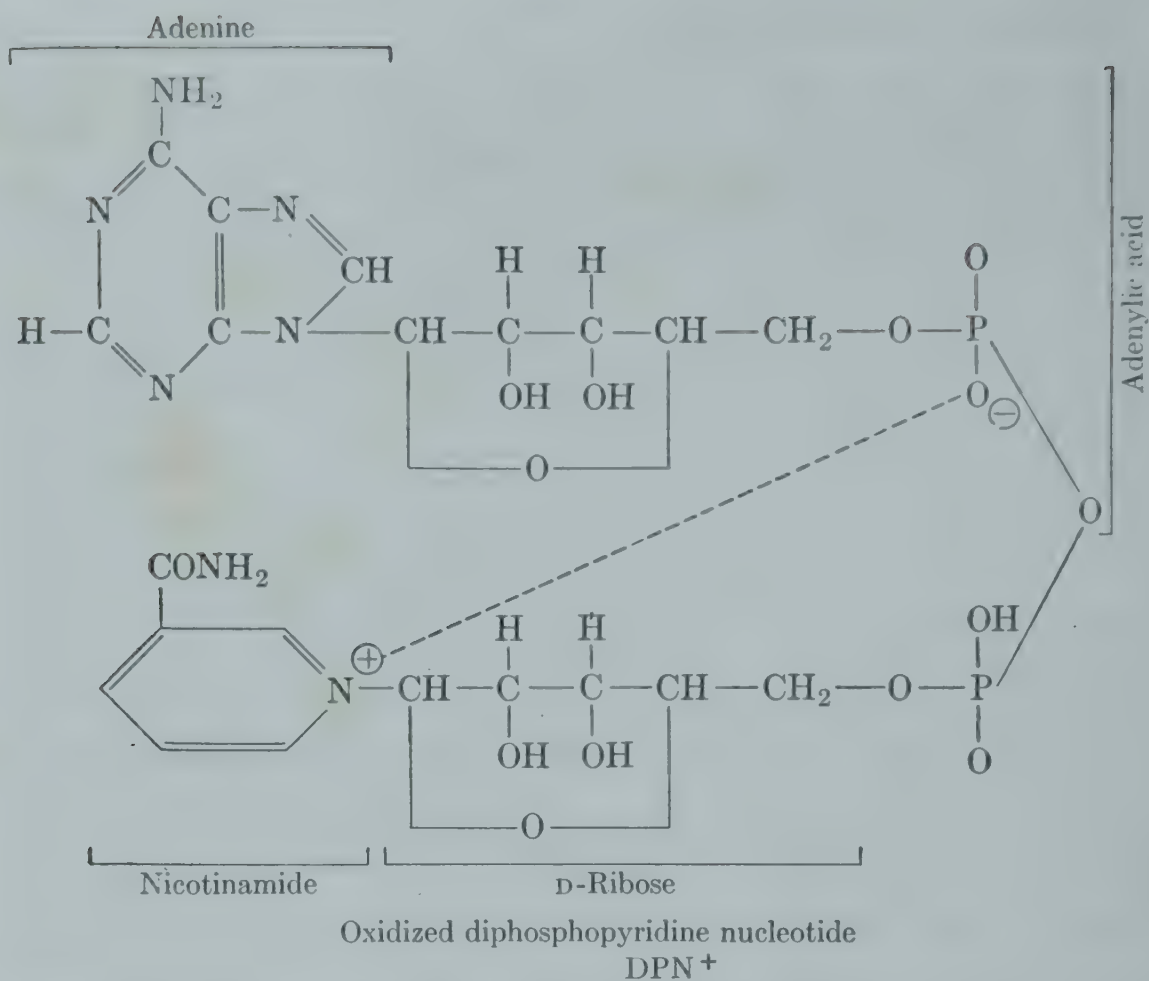
When a sample of tissue is washed exhaustively before being treated with methylene blue it may occur that even in the presence of added substrate the tissue is no longer able to bring about reduction of the dye. This might mean that the specific enzyme is soluble and has been washed away. But with most tissues addition of boiled tissue extract restores the catalytic activity, hence the enzyme protein must still be present. The same loss of activity results from prolonged dialysis and again catalytic activity is restored to the tissue suspension by boiled tissue extract. It has been shown that the preliminary washing or dialysis separates from the insoluble enzyme proteins certain small water-soluble molecules which are restored to the system when boiled tissue extract is added. Such compounds, essential to the action of an enzyme, are known as coenzymes

and are found more or less closely bound to the proteins of the majority of the oxidizing enzymes. It is usually possible by suitable procedures to separate the thermolabile enzyme proteins, or *apoenzymes*, from the thermostable prosthetic groups which serve them as coenzymes. Separation may result for example from a change in *pH* or from selective precipitation with organic solvents. As we have just seen, dialysis sometimes brings about the same result. Once separated, neither the coenzyme nor the protein alone is catalytically active, but usually activity can be restored by putting the two together.

An oxidizing enzyme then consists of a protein part in which resides the substrate specificity, and a coenzyme of low molecular weight which can be reversibly oxidized and reduced. Such a coenzyme is the flavin-adenine dinucleotide which was described in Chapter 7 as accepting hydrogen from D-amino acids. The oxidase protein which is needed for this specific oxidation is only one of several oxidizing enzymes which are active in the presence of this particular dinucleotide. Thus the same coenzyme is found to act in conjunction with one protein to oxidize an amino acid, for example, and in the presence of a different protein to oxidize a quite different substrate such as glucose. The enzymes of this group are known as the *flavoproteins*. Another group of dehydrogenases consists of specific proteins combined with somewhat similar prosthetic dinucleotides in which the flavin is replaced by the pyridine derivative, nicotinamide. These enzymes are the *pyridinoproteins*. Still a third class of oxidizing enzymes has as prosthetic group a metalloporphyrin of the heme type. Before attempting to trace the relation of these various oxidation catalysts to each other, it will be necessary to consider separately the chemistry of the three different types of coenzymes.

THE PYRIDINOPROTEIN ENZYMES

The name cozymase was used early in the century to designate the unknown essential factor which was removed from yeast juice by dialysis. This substance was eventually isolated from yeast juice by Hans von Euler of the University of Stockholm. Shortly thereafter Warburg and Christian obtained a similar compound from red blood corpuscles where it takes part in the oxidation of glucose-6-phosphate to 6-phosphogluconic acid. The yeast factor then became "coenzyme I" and the Warburg preparation from blood "coenzyme II." The two compounds proved to be very much alike in chemical composition as both yielded on hydrolysis D-ribose, adenine, nicotinamide, and phosphoric acid. They differed only in their phosphate content, coenzyme II yielding three moles of phosphoric acid per mole, while the yeast factor, coenzyme I, contained only two. The structure of the latter compound proved to be that of a dinucleotide with the two mononucleotides linked together through their phosphate groups. Since



nicotinamide is a pyridine derivative, and because of the two phosphate groups in the compound, coenzyme I is known as diphosphopyridine nucleotide. This is commonly abbreviated to DPN, while the older name becomes CoI.

The position of the third phosphate group in coenzyme II remained in doubt long after the structure of coenzyme I had been elucidated. It has now been proved that the two molecules are alike except that CoII has a third phosphate group esterified at position 2 of the ribose in the adenosine-5-phosphate part of the dinucleotide.

It is interesting to note at this point that nicotinamide is the third member of the group of B vitamins which we have found to be used in living cells for synthesis of a coenzyme. Phosphorylated thiamine is co-carboxylase; phosphorylated pyridoxal is the coenzyme of transamination, and in coenzymes I and II nicotinamide appears in a complex phosphate-containing molecule. We shall find other members of the group serving similar functions in other coenzymes.

Mechanism of Dehydrogenation by Pyridine Enzymes. Study of the chemistry of the pyridine coenzymes was facilitated by their having strong absorption bands in the ultraviolet.

In the oxidized form they show a single band at about 2600Å. When they are reduced either chemically as with hyposulfite, or enzymatically with a dehydrogenase and its substrate, a second absorption band appears at 3450Å. Absorption curves for coenzyme I in the oxidized and in the reduced form are given in Figure 14.2. Corresponding curves for coenzyme II are almost identical.

The absorption band at 2600Å can be accounted for by the known absorption spectra of the two heterocyclic rings in the molecule. Therefore when Warburg found that reduction of a simple pyridine derivative caused the appearance of a band at 3600Å he concluded that the reduction of the coenzyme which gives rise to a new band at 3450Å is in reality simply the reduction of the pyridine part of the molecule. For the simple methiodide of nicotinamide he formulated the reduction as follows, two hydrogen atoms yielding to the ring one

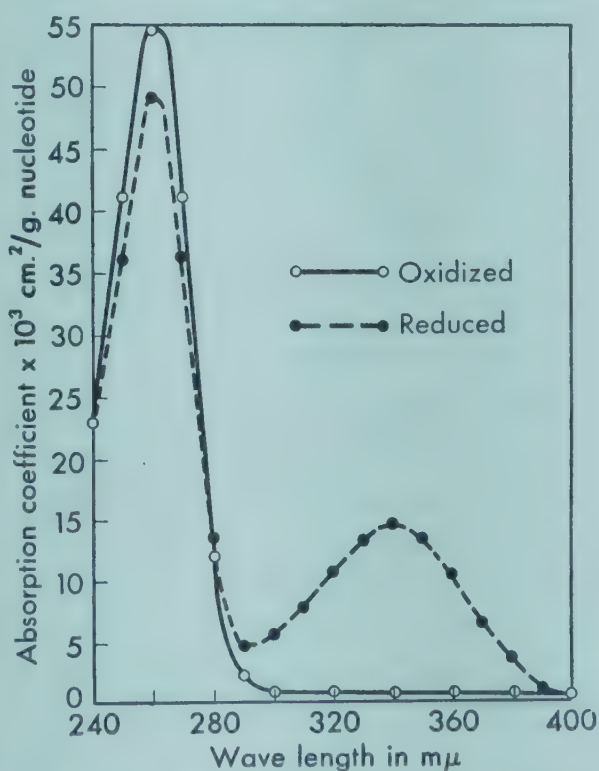
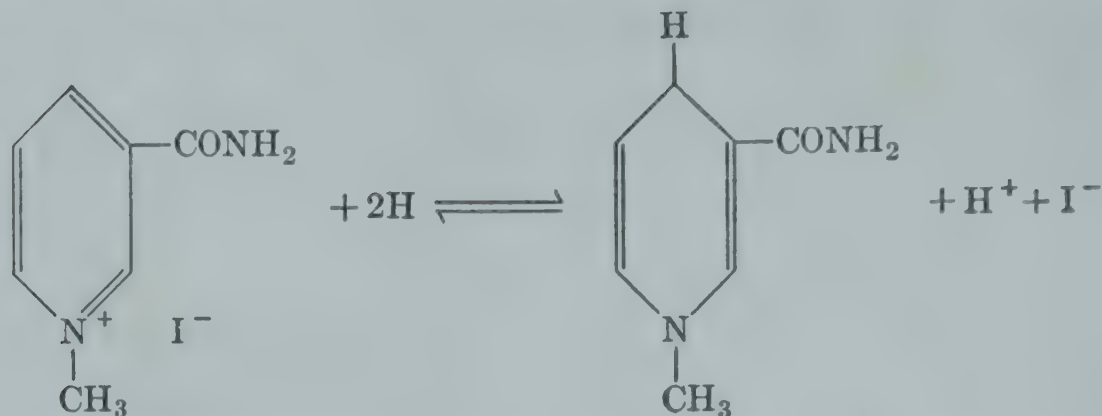
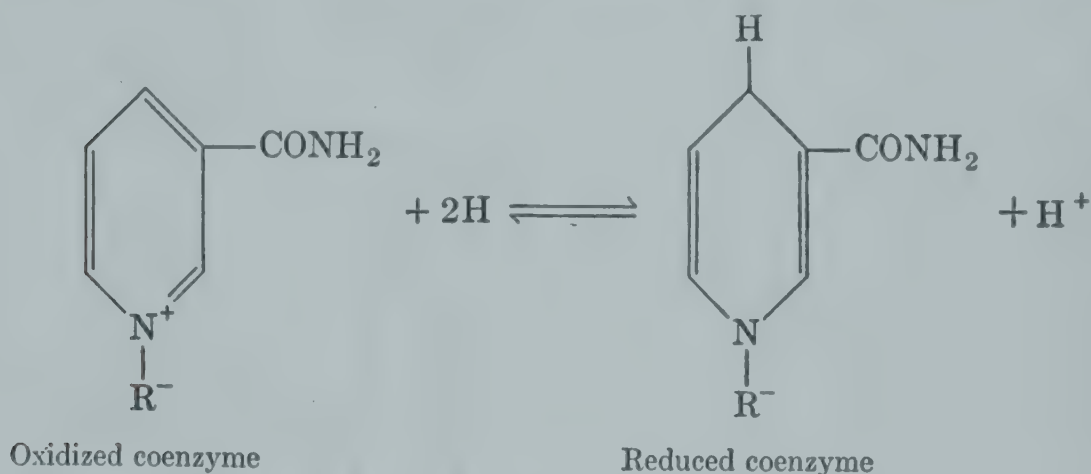


Figure 14.2. Absorption curves of oxidized and reduced diphosphopyridine nucleotide (pH 7.4). (From O. Warburg and W. Christian, *B.Z.*, 287:291, 1936.)

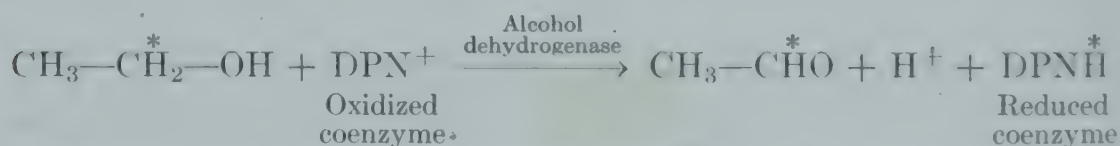
hydrogen atom and one electron, and forming in solution a hydrogen ion.



The same reaction is believed to take place when the coenzymes are reduced. In the following formulation R stands for all of the dinucleotide except the nicotinamide, the negative charge being associated with one of the phosphate groups in the coenzyme itself.



The accuracy of Warburg's formulation has been proved very recently in experiments with alcohol labeled with deuterium. This compound was used as substrate with alcohol dehydrogenase and crystalline diphosphopyridine nucleotide, and the reduced coenzyme was later isolated. The presence of deuterium in the reduced coenzyme proved the actual transfer of hydrogen from substrate to coenzyme, and ruled out the possibility that the hydrogen which attached itself to the coenzyme came from water and not from the substrate at all.



The positions of the isotopic hydrogens are indicated by the starred atoms.

Although coenzymes I and II both act in conjunction with several different proteins, they are not usually interchangeable. Those enzymes which use CoI are in general inactive or nearly so in the presence of CoII, and vice versa. In Table 14-I are listed the dehydrogenases which

have been most completely characterized. Although any acid substrates are present as ions in the physiological pH range, the dehydrogenases are named as though the acids themselves were acted upon. Thus we speak of *lactic dehydrogenase* or *malic dehydrogenase* rather than lactate or malate dehydrogenase. The others are named directly from their substrates, as *glucose dehydrogenase* or *triosephosphate dehydrogenase*. It should be noted that the rule about coenzyme specificity is not without exceptions, since a few enzyme proteins can make use of either one. This is true, for example, of both glutamic and glucose dehydrogenases. Usually however, even when an enzyme can use either of the two coenzymes it acts much less rapidly with one than with the other.

At the end of the table are listed four dehydrogenases which so far have resisted separation into apoenzyme and coenzyme. Whether they will always remain in this isolated position, or will prove to have coenzymes very firmly bound is still an open question. Of these four, succinic dehydrogenase is the most important and most widely distributed.

TABLE 14-I. REPRESENTATIVE DEHYDROGENASES

Substrate	Reaction Catalyzed	Source of Enzyme
I. Enzymes which Use DPN (Coenzyme I)		
Alcohol	$\text{CH}_3\text{—CH}_2\text{OH} \rightleftharpoons \text{CH}_3\text{—CHO}$	Bacteria, liver, yeast, seeds, leaves
L(+)-Malic acid ^a	$\begin{array}{ccc} \text{CHOH—COOH} & \rightleftharpoons & \text{CO—COOH} \\ & & \\ \text{CH}_2\text{—COOH} & & \text{CH}_2\text{—COOH} \\ \text{Malic acid} & & \text{Oxalacetic acid} \end{array}$	Many animal tissues, yeast, bacteria, roots and seeds
L(+)-Lactic acid ^a	$\text{CH}_3\text{—CHOH—COOH} \rightleftharpoons \text{CH}_3\text{—CO—COOH}$ Lactic acid Pyruvic acid	Many animal tissues Yeast
L(+)-Glutamic acid	$\begin{array}{ccc} \text{CH}_2\text{—COOH} & & \text{CH}_2\text{—COOH} \\ & & \\ \text{CH}_2 & \rightleftharpoons & \text{CH}_2 \\ & & \\ \text{CH(NH}_2\text{)—COOH} & & \text{C(=NH)—COOH} \\ \text{Glutamic acid} & & \text{Imino cpd.} \end{array}$	Liver: uses CoI or CoII Yeast: uses CoII Plants: uses CoI
α-Glycerophosphate (soluble)	$\begin{array}{ccc} \text{CH}_2\text{O—ph} & & \text{CH}_2\text{O—ph} \\ & & \\ \text{CHOH} & \rightleftharpoons & \text{C=O} \\ & & \\ \text{CH}_2\text{OH} & & \text{CH}_2\text{OH} \\ \alpha\text{-Glycerophosphate} & & \text{Dihydroxyacetone phosphate} \end{array}$	Muscle Oat seedling
Triosephosphate	$\begin{array}{ccc} \text{CH}_2\text{O—ph} & & \text{CH}_2\text{O—ph} \\ & & \\ \text{CHOH} & \rightleftharpoons & \text{CHOH} \\ & & \\ \text{CHO} & & \text{COOH} \\ \text{3-Phosphoglycerose} & & \text{3-Phosphoglyceric acid} \end{array}$	Bacteria Muscle Yeast Plants
Glucose	$\text{Glucose} + \text{H}_2\text{O} \rightleftharpoons \text{Gluconic acid}$	Liver: uses CoI or CoII
β-Hydroxybutyric acid	$\text{CH}_3\text{—CHOH—CH}_2\text{—COOH} \rightleftharpoons \text{CH}_3\text{—CO—CH}_2\text{—COOH}$ β-Hydroxybutyric acid Acetoacetic acid	Heart, kidney, liver

^a Can use CoII but much more slowly.

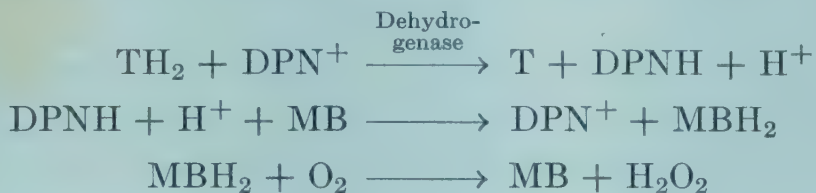
[Continued on next page]

TABLE 14-I. REPRESENTATIVE DEHYDROGENASES (Continued)

Substrate	Reaction Catalyzed	Source of Enzyme
II. Enzymes which Use TPN (Coenzyme II)		
Glucose-6-ph	$ \begin{array}{ccc} \text{CHO} & & \text{COOH} \\ & & \\ (\text{CHOH})_4 & \rightleftharpoons & (\text{CHOH})_4 \\ & & \\ \text{CH}_2\text{O-ph} & & \text{CH}_2\text{O-ph} \\ \text{Glucose-6-phosphate} & & \text{6-Phosphogluconic acid} \end{array} $	Red blood cells Yeast
6-Phosphogluconic acid	$ \begin{array}{ccc} \text{COOH} & & \text{CHO} \\ & & \\ (\text{CHOH})_4 & \rightarrow & (\text{CHOH})_3 + \text{CO}_2 \\ & & \\ \text{CH}_2\text{O-ph} & & \text{CH}_2\text{O-ph} \\ \text{6-Phosphogluconic acid} & & \text{Ribose-5-phosphate} \end{array} $	Yeast Most mammalian tissue except muscle
Glutamic acid	See Section I	
Isocitric acid	$ \begin{array}{ccc} \text{CH}_2\text{—COOH} & & \text{CH}_2\text{—COOH} \\ & & \\ \text{CH—COOH} & \rightarrow & \text{CH—COOH} \\ & & \\ \text{CHOH—COOH} & & \text{CO—COOH} \\ \text{Isocitric acid} & & \text{Oxalosuccinic acid} \end{array} $	Animal and plant tissues
Glucose	See Section I	
III. Dehydrogenases which Require No Coenzyme		
Succinic acid	$ \begin{array}{ccc} \text{CH}_2\text{—COOH} & & \text{CH—COOH} \\ & \rightleftharpoons & \\ \text{CH}_2\text{—COOH} & & \text{HOOC—CH} \\ \text{Succinic acid} & & \text{Fumaric acid} \end{array} $	Widely distributed among plants, animals and microorganisms
Lactic acid	See Section I	The yeast enzyme requires no coenzyme
α -Glycerophosphate (insoluble)	See Section I	Animal tissues Bacteria
Choline	$ \begin{array}{ccc} \text{CH}_2\text{OH} & & \text{CHO} \\ & \rightleftharpoons & \\ \text{CH}_2\text{—N}\equiv(\text{CH}_3)_3 & & \text{CH}_2\text{—N}\equiv(\text{CH}_3)_3 \\ & & \\ \text{OH} & & \text{OH} \\ \text{Choline} & & \end{array} $	Liver

Much of the work on oxidizing enzymes has been done on mammalian tissue, yeasts, and bacteria. It has been found that the same dehydrogenases occur in such different tissues as the very specialized cells of mammalian liver, on the one hand, and many unicellular organisms on the other. Furthermore, although the survey of plant enzymes is still far from complete, many of the familiar dehydrogenases have been found also in the tissues of the higher plants. It therefore seems probable that the transfer of hydrogen to a pyridine coenzyme is an oxidative mechanism common to all aerobic cells.

Summary. The oxidation of many substrate molecules begins in the presence of a specific dehydrogenase protein with the transfer of hydrogen from a metabolite to a pyridine dinucleotide. This presumably takes place when coenzyme and substrate form a triple complex with the enzyme protein. In order that a molecule of coenzyme may continue to function as a hydrogen acceptor it must be promptly re-oxidized by transferring this hydrogen to some other reducible substance. In the artificial systems which include methylene blue, the dye acts as acceptor and is bleached anaerobically to its leuco form. If this type of experiment is carried out aerobically, there is a continuous uptake of oxygen which simulates normal respiration. The reduced methylene blue under these conditions passes on its hydrogen to oxygen and so acts as an intermediate carrier of hydrogen from primary pyridine acceptor to the terminal acceptor, oxygen. Letting TH_2 stand for any oxidizable tissue substance, the process may be represented as taking place in a series of steps as follows:



In this process both the coenzyme and the dye are repeatedly reduced and then oxidized, each complete cycle leaving them in the oxidized form, ready to accept another pair of hydrogens.

But if a purified dehydrogenase is treated with its substrate in the absence of methylene blue, there is no oxygen uptake. The appearance of the characteristic absorption band at 3450\AA proves that the coenzyme has been reduced, but there the reaction stops. This means that the reduced coenzymes are not capable of reacting directly with molecular oxygen. Since tissue slices do take up oxygen, there must be in the intact cell some sort of intermediate carrier which acts as methylene blue does in the artificial system to bridge the gap between the reduced pyridine compound and oxygen. Several such substances have been found among the other groups of oxidizing enzymes. Together they constitute the hydrogen transport system, a sort of bucket brigade in which the pyridine coenzymes serve as the first bucket. Of these intermediate substances the first to be recognized were those involved in the final stages of the reaction, where activation of oxygen results in formation of water. These are the iron porphyrin enzymes which will be considered in the following section.

THE IRON PORPHYRIN ENZYMES

We have already noted that as early as 1920 Warburg was searching for an *Atmungsferment* or respiratory enzyme in which the active agent would be an organic compound of iron. Although he did not isolate such

a compound, his early work should be noted at this point because of his fundamental contributions to oxidative theory, and even more because of its brilliant indirect approach to a problem which could not at that time be attacked directly.

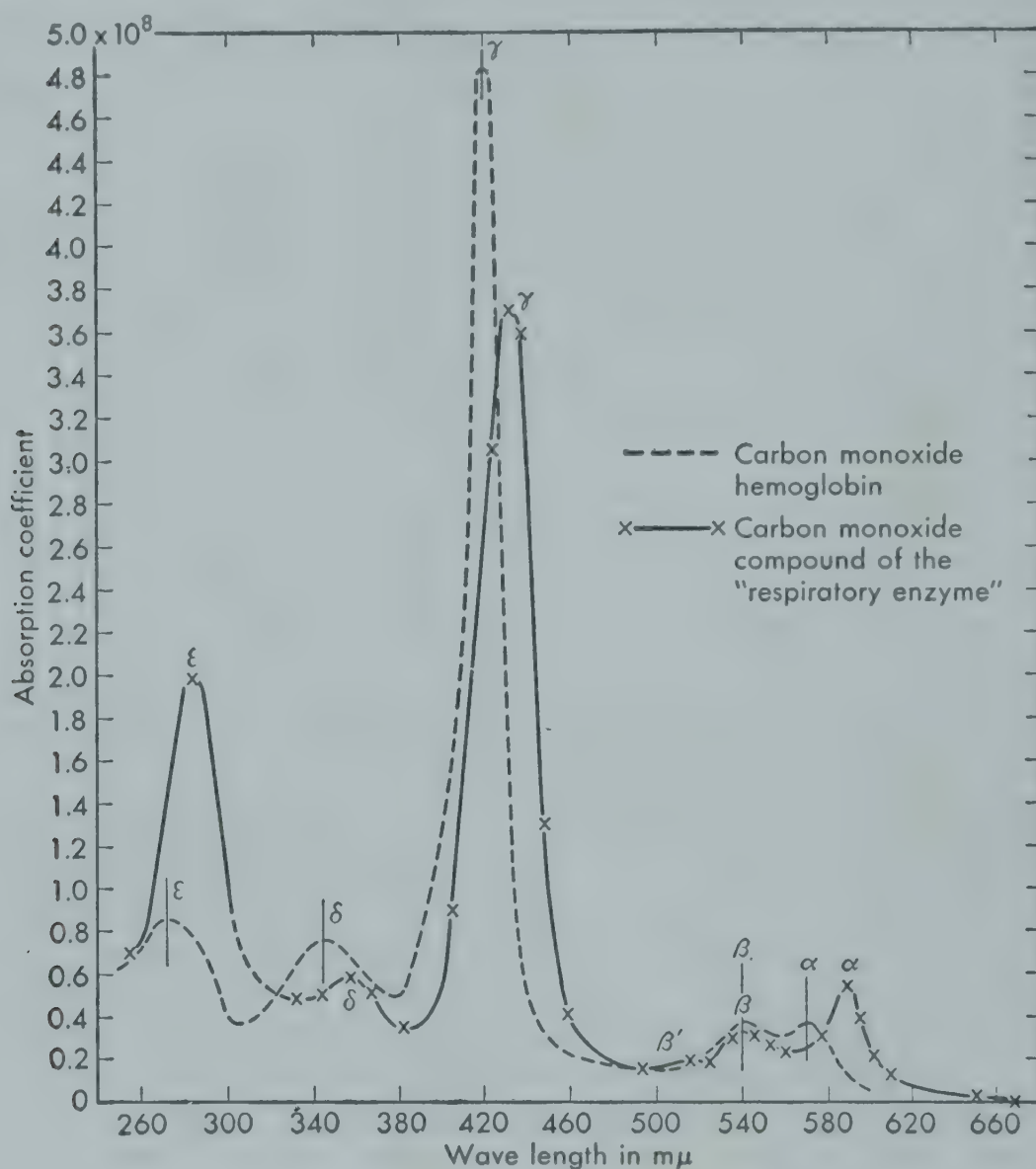


Figure 14.3. Absorption spectra of carbon monoxide hemoglobin and of the carbon monoxide compound of Warburg's "Atmungsferment." (From O. Warburg, *Z. Angewandte Chemie*, 45:1, 1932.)

Working with enzyme inhibitors, he found that in the dark the respiration (oxygen uptake) of living cells was almost completely abolished by high concentrations of carbon monoxide, and that this inhibition was reversed by light. It was known that carbon monoxide forms complexes, known as metal carbonyls, with many heavy metals including iron and that these compounds decompose in the light. Furthermore, the iron of hemoglobin was known to unite similarly with carbon monoxide. Warburg therefore reasoned that the inhibition of respiration by carbon monoxide resulted from combination of this gas with the iron in a respiratory ferment,

and that dissociation of the carbon monoxide complex by light restored the activity of the enzyme.

The effectiveness of light in bringing about reactivation of the oxidizing system proved to depend upon its wave length. In one experiment illumination of monoxide-poisoned yeast with ultraviolet light resulted in almost no additional oxygen uptake, while blue light (4360Å) brought about a fourfold increase. Since light must be absorbed in order to act upon a compound, this observation provided a means of determining the absorption spectrum of a purely hypothetical cell constituent! In Figure 14.3 the effectiveness of light in reversing the carbon monoxide inhibition of respiration is plotted against its wave length. On the reasonable assumption that a large reversal of inhibition results from a high absorption of light of that wave length, this curve then gives the absorption spectrum of the enzyme-carbon monoxide complex. From the point of view of Warburg's thesis this curve was significant because it proved to resemble so strongly a similar curve obtained with carbon monoxide hemoglobin, in which the carbon monoxide is known to be attached to iron. This similarity was therefore interpreted as evidence that a respiratory enzyme would in all probability prove to be an iron tetrapyrrole compound.

Discovery of the Cytochromes. As early as 1886 it had been reported that a substance present in muscle exhibited four characteristic absorption

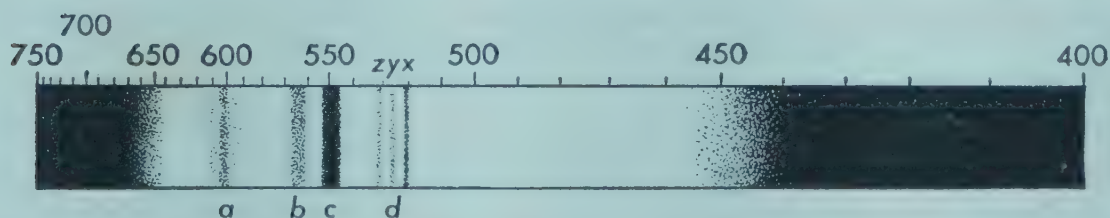


Figure 14.4. Absorption spectrum of cytochrome in thoracic muscles of a bee. A layer of muscle 0.65 mm. thick was examined in a Zeiss microspectroscope. (From D. Keilin, *Proc. Roy. Soc., B* 98:312, 1925.)

bands in the visible region. The compound was never isolated and was eventually completely forgotten.

In 1925 Keilin² rediscovered this substance, named it "cytochrome" and showed that it was present in every type of aerobic cell or tissue examined. This demonstration is remarkably simple with organisms which do not contain hemoglobin, which also absorbs light in the visible region. For example, a yeast suspension or the thoracic muscles of a honey bee pressed on a microscope slide show readily the four characteristic absorption bands which are represented in Figure 14.4. However, when oxygen is bubbled through the yeast the bands disappear, to be replaced by a very low, diffuse absorption. If the oxygen is removed by a stream of nitrogen the four bands reappear. These experiments indicate that the cytochrome

² David Keilin, Professor of Biology at Cambridge University, has been for the past quarter century a distinguished contributor in the field of enzyme chemistry.

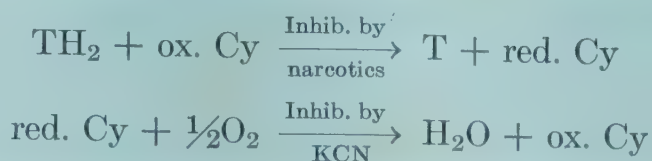
can be reversibly oxidized and reduced, and that it is the reduced form to which the sharp absorption bands are due. The positions and relative intensities of these bands differed slightly from tissue to tissue but were remarkably constant for a wide variety of organisms as is indicated in Figure 14.5. Occasionally one would be absent. These irregularities proved to be due to the fact that "cytochrome" is really a mixture of several different but very similar components. The four absorption bands represent a summation of two individual bands per component, some of which overlap.

610	600	590	580	570	560	550	540	530	520	
6046	a Bee: wing muscles			5665	b	5502	c		5210	d
6038	Dytiscus: wing muscles			5664		5495			5205	
6046	Galleria: wing muscles			5657		5495			5200	
6035	Helix: radula muscles			5650		5495			5200	
6040	Frog: heart muscle			5660		5500			5205	
6045	Guinea pig: heart muscle			5662		5500			5205	
6035	a Yeast cells			5645	b	5490	c		5190	d

Figure 14.5. Positions of the four main absorption bands of the cytochromes in various organisms. (From D. Keilin, *Proc. Roy. Soc., B* 98:312, 1925.)

Two simple experiments with respiratory inhibitors clarified the role which the cytochromes play in cellular oxidation. A yeast suspension in which the cytochrome was in the reduced form was treated with potassium cyanide and then oxygenated. The bands of the reduced cytochrome failed to disappear. But if cyanide was added to a suspension in which the pigment was already in the oxidized form an immediate reduction resulted as shown by the appearance of the characteristic bands of reduced cytochrome. These two results indicate that there is in the cells a mechanism for the reduction of oxidized cytochrome which is not influenced by the presence of cyanide, and a second which brings about oxidation of the reduced cytochrome and is inhibited by cyanide. In similar experiments, such narcotic enzyme inhibitors as alcohol or ethyl urethane prevented the reduction of the oxidized form, but did not interfere with the oxidation of reduced cytochrome. Thus cytochrome was shown to take part in an enzymic, cyclical process in the cells, being reduced by hydrogen donors and reoxidized by molecular oxygen. The enzymes responsible for the reduction are inhibited by narcotics but not by cyanide, while the reverse is true of the oxidizing system. This points to a fundamental difference in the catalysts involved, and suggests that the one which brings about the reaction with molecular oxygen may contain iron, since it is

sensitive to cyanide. Allowing ox. Cy to stand for the oxidized cytochrome and red. Cy to stand for the reduced form, the two reactions in which this compound is involved may be represented:



Keilin recognized the existence of three different components in cytochrome, and called them cytochromes *a*, *b*, and *c*. At least two others

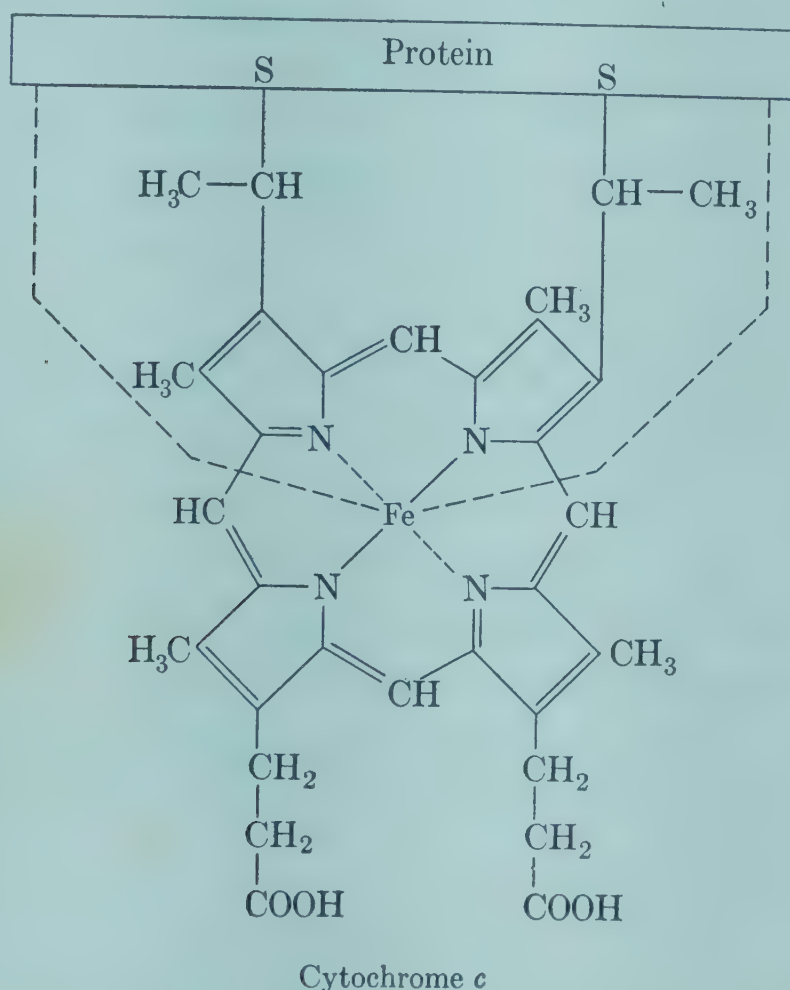


Figure 14.6. Diagram of the tetrapyrrole of cytochrome with its bonds to the protein part of the enzyme. The links from the ethyl groups go to cysteine residues in the protein.

have been identified, and it is probable that there are nine or ten individuals of closely related composition. Keilin succeeded in isolating the *c* component and it has since been purified and exhaustively investigated by many different workers. It proves to be a colored conjugated protein the prosthetic group of which is closely related to heme. It has the very low molecular weight of 13,000 and one iron porphyrin group per molecule. The porphyrin proves to be very firmly linked to the protein. Theorell, whose formula for cytochrome *c* is given in Figure 14.6, speaks of the heme in cytochrome as being "built into a crevice in the protein molecule,"

where its iron atom is protected from autoxidation. Of the other cytochromes little is known except that they are iron porphyrin proteins with heme or a slightly modified heme as prosthetic group.

Oxidation and Reduction of the Cytochromes. When a cytochrome is oxidized its iron changes from the ferrous to the ferric condition. The oxidized and reduced modifications are therefore often referred to as ferricytochromes and ferrocytochromes. In their normal functioning in the cells the cytochromes thus differ from hemoglobin, in which the iron remains in the ferrous condition unless it is oxidized chemically.

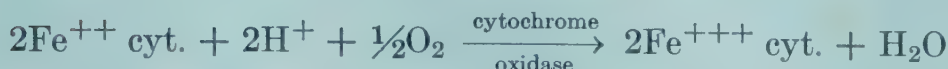
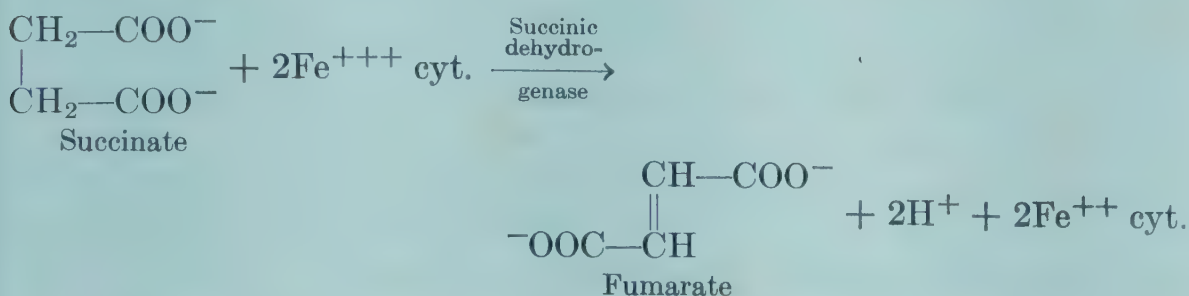
Purified cytochrome *c* is readily reduced by mild reducing agents, but the reduced ferrocytochrome cannot be reoxidized by molecular oxygen. Since this same substance in a cell suspension reacts readily with oxygen there must be in the cell some catalyst which mediates the reaction. This catalyst proves to be an enzyme, known as *cytochrome oxidase*, which is also an iron pyrrole protein. It is closely associated with insoluble parts of the cells, but has recently been obtained free of most of the cell debris and greatly concentrated.

Cytochrome oxidase is extremely sensitive to cyanide, hydrogen sulfide, and sodium azide. It is furthermore inhibited by carbon monoxide and this inhibition is reduced in strong light. These are of course the properties of Warburg's *Atmungsferment*. There is in fact much evidence that this enzyme which brings about the oxidation of cytochrome by molecular oxygen is indeed that one which was the object of Warburg's earlier investigations. It is an iron porphyrin compound, closely related chemically to the other cytochromes. In fact Keilin named it cytochrome *a₃* to indicate its relationship to the original *a* component. Thus with this enzyme the usual order of events has been reversed. Warburg had deduced some of its chemical and physical properties before there was any sure indication of its existence, and even then a good many years were to pass before it was finally isolated.

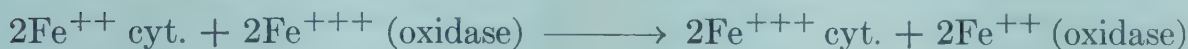
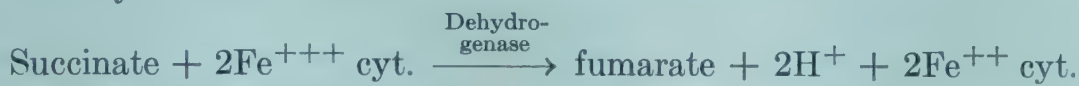
Since the only change in cytochrome when it is reduced is a change in the valence of iron, it may seem strange that this electron change leads to formation of water as shown in the equations on page 471. This is explained by assuming that each hydrogen reduces the cytochrome by donating an electron and remaining in the vicinity as a hydrogen ion which can be called on for formation of water. Under these conditions it may be said that identification of cytochrome oxidase clarified the final oxidative step in which hydrogen from a metabolite is transferred from reduced cytochrome to molecular oxygen. But there was still no light on the pathway by which hydrogen reaches and reduces cytochrome, since neither coenzyme I or II donates hydrogen directly to cytochrome.

The simplest complete system in which cytochrome is reduced by a metabolite is the one in which succinate is oxidized to fumarate by cell-free enzymes. It will be remembered that succinic dehydrogenase is an enzyme

which has no known coenzyme. When purified cytochrome *c* in its oxidized form is added anaerobically to succinate and its dehydrogenase, the absorption bands of reduced cytochrome appear. If oxygen is then admitted and cytochrome oxidase added there ensues a rapid oxygen uptake during which the cytochrome is reoxidized. The sequence of reactions involved in these two steps may be represented by equations, allowing $\text{Fe}^{++}\text{cyt.}$ to stand for ferrocytochrome and $\text{Fe}^{+++}\text{cyt.}$ for the oxidized form.



Since cytochrome oxidase is itself an autoxidizable compound of the cytochrome group, the reaction may be more completely outlined by including one more step and allowing $\text{Fe}^{+++}(\text{oxidase})$ to stand for the oxidized form of the cytochrome oxidase.



Here again is a cyclic process in the course of which each of the iron pyrrole compounds is returned to the oxidized condition. In this way small amounts of these compounds can act again and again in hydrogen transport.

Even for this most direct of the oxidations by way of cytochrome, this formulation undoubtedly oversimplifies. It takes no account of there being at least three cytochromes besides the oxidase, and this for the best of reasons, that their individual roles are not yet clear. Since the oxidase reacts best with cytochrome *c*, these two are believed to be the last two compounds through which electrons pass (in a fashion which cannot be exactly defined) until they, accompanied by their protons, are finally donated to oxygen with formation of water. It is probable that these electrons reduce several other cytochromes before they reach cytochrome *c*. But until the exact course of the reactions has been determined, it seems best to group the cytochromes together, recognizing that the oxidations and reductions may in some tissues involve them all in sequence, and may in others be concerned with only one or two.

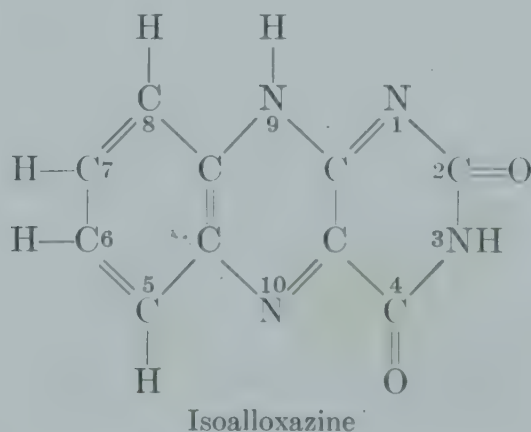
Significance of the Cytochromes. Keilin's first paper indicated that the cytochromes were very widely distributed. Later work has shown that

they are present in many plant as well as animal tissues and in the cells of all microorganisms except the strict anaerobes. In animals, yeasts, and bacteria they are believed to constitute the chief terminal oxidizing system, to which nearly all others must donate hydrogens in order that they may ultimately react with molecular oxygen and form water. This is indicated for example by the fact that cyanide which inhibits cytochrome oxidase very strongly, also very nearly abolishes the respiration of most cells. In the plants the cytochromes may not fulfill so unique a function, since there are other enzymes which are capable of linking the cell substrates with oxygen (see pp. 482-484). Nevertheless, cytochrome *c* is present in many plant cells. It has been isolated from wheat germ in sufficient amount to account for the observed respiration of the tissue. A sample of cytochrome oxidase from the same source is apparently identical with that from animal heart muscle. These facts would indicate that in at least some plant cells cytochrome acts as the terminal oxidase system, as it does elsewhere in nature.

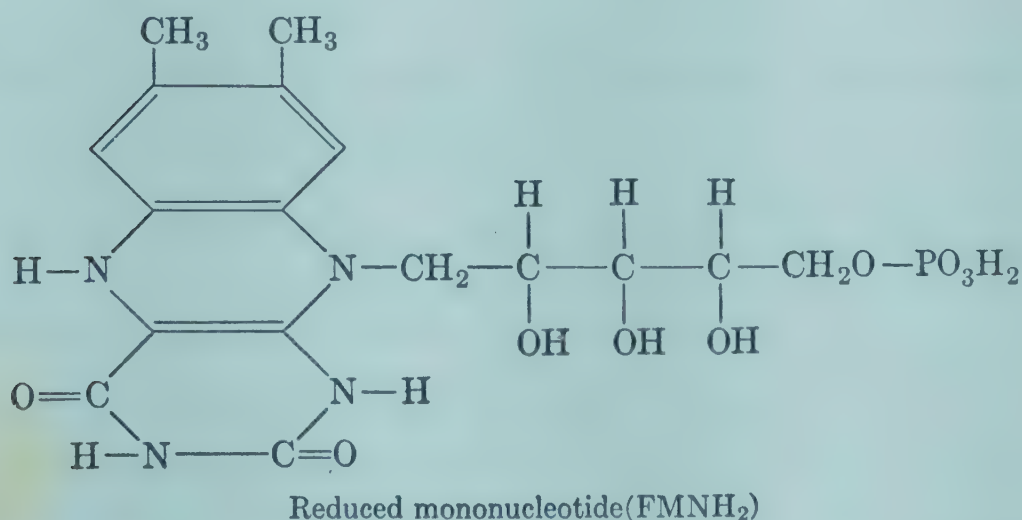
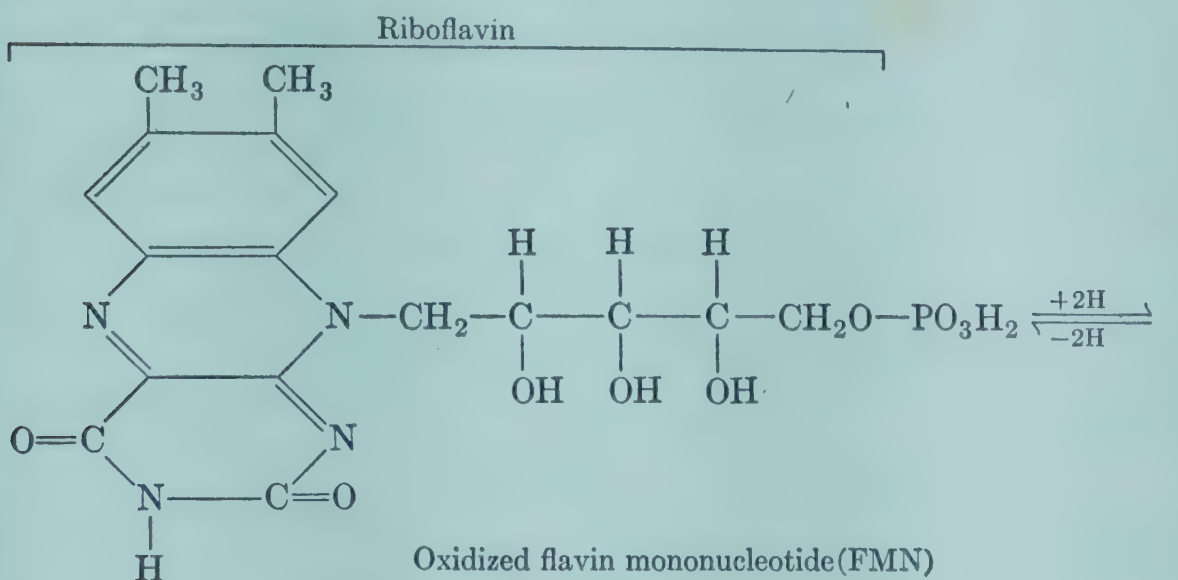
THE FLAVOPROTEIN ENZYMES

Although, as we have just seen, the cytochromes can accept hydrogens directly from succinate, there are few of the common metabolites which act in this way. For most of the compounds which are oxidized by living cells the pyridine coenzymes are obligatory hydrogen acceptors. It might have been expected that the reduced pyridine compounds would then hand on their hydrogens to a cytochrome. But work with purified enzymes soon showed that this could not be done. The reduced pyridine dinucleotides can be oxidized no more by cytochrome than by molecular oxygen. There must therefore be still another link in the hydrogen transport system. This intermediate carrier is now believed to be one of the conjugated proteins known as the *flavoproteins*.

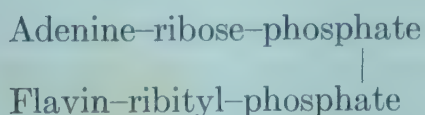
The Flavin Coenzymes. The flavoproteins consist of a protein apoenzyme combined with a soluble coenzyme which includes the B vitamin known as lactoflavin or riboflavin or vitamin B₂. This vitamin is a derivative of a complex heterocyclic compound known as isoalloxazine.



Riboflavin is a 6,7-dimethyl derivative of isoalloxazine with which the sugar alcohol, ribitol, has been condensed at position 9. When this compound is further modified by phosphorylation at carbon 5' of the ribitol, the resulting substance is one of the two known flavin coenzymes. It is usually referred to as a flavin mononucleotide (FMN) in spite of the fact that the sugar component is not a pentose as in a true nucleotide but the related pentahydroxy alcohol.



The second flavin coenzyme is the flavinadenine dinucleotide (FAD) which has been used several times as an example of an oxidizing coenzyme. The molecular pattern of this substance is like that of coenzyme I except that riboflavin replaces the nicotinamide-ribose complex.



In Table 14-II are listed the best characterized of the enzymes which require a flavin derivative as coenzyme. The members of the first group

TABLE 14-II. SOME REPRESENTATIVE FLAVOPROTEINS

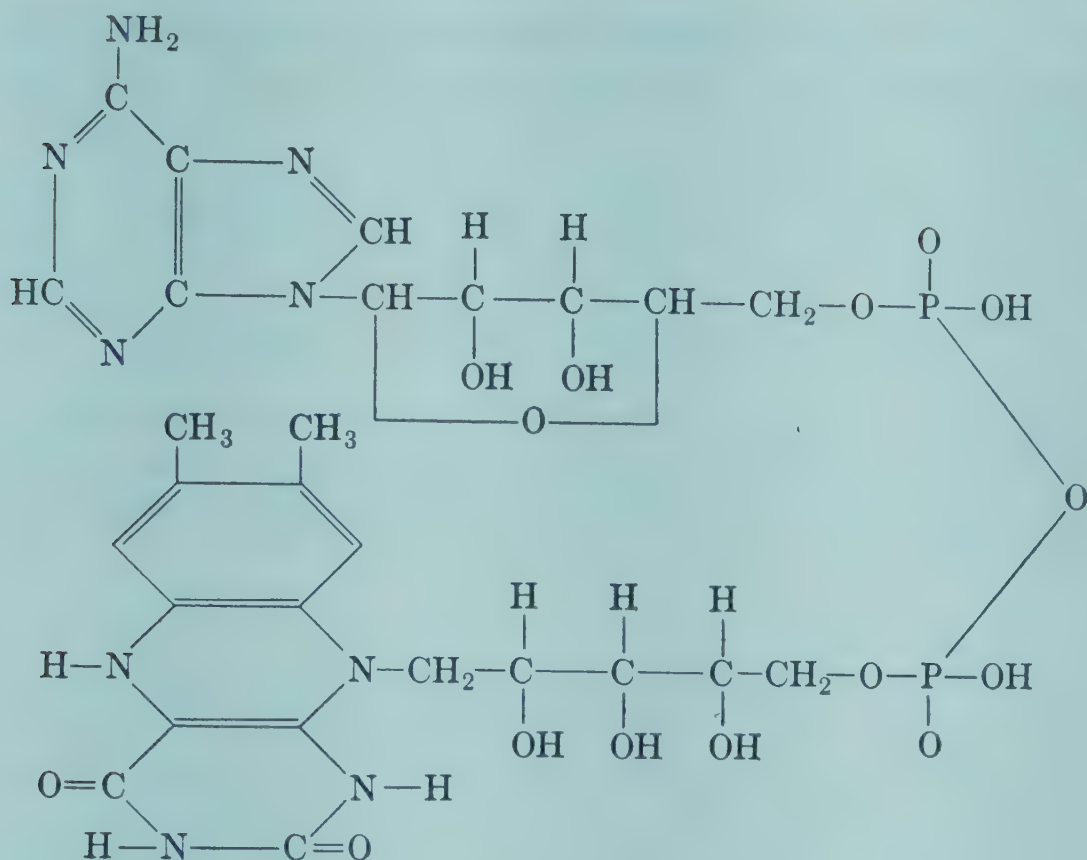
Name	Substrate		Oxidation Product of Hydrogen	Source of Enzyme
	Reduced Form	Oxidized Form		
I. <i>Enzymes which Use Flavin Mononucleotide (FMN)</i>				
Old yellow enzyme	CoI—H ₂ CoII—H ₂	CoI CoII	H ₂ O ₂	Yeast
Cytochrome <i>c</i> reductase	CoII—H ₂	CoII	H ₂ O, via the cytochromes	Yeast
L-Amino acid ^a oxidase	R—CH—COOH NH ₂ L-Amino acid	R—C—COOH NH Imino acid ^b	H ₂ O ₂	Kidney Neurospora (a mold) Viper venom
	R—CHOH—COOH Hydroxy acid	R—CO—COOH Keto acid		
II. <i>Enzymes which Use Flavin Dinucleotide (FAD)</i>				
D-Amino acid oxidase	R—CH—COOH NH ₂ D-Amino acid	R—C—COOH NH Imino acid ^b	H ₂ O ₂	Kidney Neurospora
Glucose oxidase (notatin)	Glucose	Gluconic acid	H ₂ O ₂	Molds
Xanthine oxidase ^a	Various purines R—CHO	Uric acid R—COOH	H ₂ O ₂	Milk Some liver and kidney tissue
Glycine oxidase	CH ₂ —COOH NH ₂	CH—COOH ^b NH	H ₂ O ₂	Kidney

^a Note the dual specificities.^b The imino compounds all react further with water to replace the =NH group with =O, forming aldehyde or keto groups and NH₃.

in the list use the mononucleotide, while the others are conjugated with the dinucleotide.

As with the pyridine coenzymes, the activity of the flavin coenzymes depends upon the reversible oxidation-reduction of one of the heterocyclic groups. With this coenzyme both hydrogens become attached to the rings as shown in the formulation given for the mononucleotide. Exactly the same reaction takes place when the dinucleotide acts as oxidative coenzyme.

The Link Between Enzyme Systems. The first flavoprotein enzyme to be isolated was the one which is now known as "Warburg's old yellow enzyme." It was isolated by Warburg and Christian in 1932 and proved to have as prosthetic group the flavin mononucleotide. The oxidized form of this flavin compound can be reduced by reduced coenzyme II, but once reduced, it is itself reoxidized too slowly by oxygen for the reaction to be biologically significant. It is not reoxidized at all by the cytochromes.

Reduced flavinadenine dinucleotide (FADH₂)

and so could not act as the urgently sought intermediate between the reduced pyridine coenzymes and the cytochrome system.

Meantime several similar yellow-colored enzymes had been obtained from various animal tissues, all of them having the flavin dinucleotide as prosthetic group. But still, although these also were able to oxidize one or other of the reduced pyridine compounds, they reacted even more slowly than did the old yellow enzyme with oxygen. They also failed entirely to reduce cytochrome. Finally, about 1939 Haas and Horecker in Hogness' laboratory at the University of Chicago isolated from yeast a new and very active flavoprotein which not only oxidized reduced TPN much more rapidly than any of the older yellow enzymes, but was in turn itself rapidly reoxidized by cytochrome *c*. This enzyme, known as *cytochrome reductase*, has as prosthetic group the flavin mononucleotide originally discovered as coenzyme for the old yellow enzyme. Kinetic studies indicate that when it acts as a link in the hydrogen transport system the specific protein of the reductase forms a quadruple complex in which it is linked simultaneously to the reduced pyridine enzyme, the flavin mononucleotide, and one of the cytochromes. The hydrogens (or electrons) can thus be transferred from one carrier to another while all are under the catalytic influence of the reductase protein. Thus at last we are possessed of a complete system of hydrogen transport, at least for those substrates which can reduce coen-

THE MAJOR OXIDATIVE PATHWAYS FOR HYDROGEN

The enzymes which are known to bring about formation of water in living cells are few. Of these the one of major importance is cytochrome oxidase which mediates oxidation of reduced cytochrome *c* by molecular oxygen. In most animal tissues this reaction is believed to account for the major part of the oxygen uptake. The paths by which hydrogen is moved from metabolite to this final acceptor vary in complexity. There may be involved a relatively simple reaction, as in the oxidation of succinate, or the hydrogens may be transferred by way of the complete hydrogen transport system. Whatever the intermediate stages, the final product when hydrogens are oxidized through the cytochromes is water. If we may judge by the number of known pyridine coenzymes the typical oxidative pathway for the majority of the tissue substrates is by way of coenzyme I or II, thence to a flavin, and finally to cytochrome.

Except for cytochrome reductase the flavoproteins seem to be concerned with oxidations which are off the main pathway. Many of these enzymes accept hydrogens directly from their respective substrates and in the reduced form are themselves autoxidizable. But, as with the amino acid oxidases, this oxidation gives rise not to water but to hydrogen peroxide. Although this substance is toxic when present in any great concentration, we shall see that the cells have more than one way of coping with it, hence it is not impossible that it is one product of normal respiratory activity. It is also possible that in the cells the reduced flavin enzymes do not react directly with oxygen at all, but pass on their hydrogens to some still unknown oxidative factor. At present it is not possible to assign to any of the flavin enzymes except cytochrome reductase a definite place in the oxidative scheme.

Miscellaneous Oxidizing Enzymes

There are a number of enzymes which catalyze oxidative reactions other than those of hydrogen transport. Some of these make it possible for the cells to use hydrogen peroxide as an oxidizing agent. Others are true oxidases in that they activate molecular oxygen to react with various metabolites. There is some evidence that these latter may act as terminal oxidases in plants.

CATALASE: AN IRON PYRROLE ENZYME

The existence of the enzyme *catalase* which catalyzes the decomposition of hydrogen peroxide to water and oxygen has long been known. It has now been obtained in crystalline form from animal tissues and bacteria and has been found to be widely distributed in the plant kingdom also. The enzyme consists of a specific protein combined with three or four

heme residues in which the iron is in the ferric state. Its molecular weight is approximately 250,000.

For many years it was assumed that the function of catalase in the cells is the destruction of any hydrogen peroxide which is formed in the course of metabolism. In view of the prevalence of flavin enzymes which lead to the formation of hydrogen peroxide *in vitro*, this may well be one major use for catalase. But it has also been shown that in the presence of catalase hydrogen peroxide will bring about oxidation of certain organic alcohols or of nitrite to nitrate. An enzyme which activates a peroxide to oxidize other molecules is said to be exerting a *peroxidase* function.

In whichever of its two functions catalase is engaged it begins by forming an enzyme substrate compound with hydrogen peroxide.



This compound of the familiar ES type may then react with a second molecule of hydrogen peroxide to form water and oxygen. In such a reaction the second peroxide molecule is said to act as a donor of hydrogen, which unites with one of the two oxygen atoms in the original substrate peroxide, forming water, and setting free its own oxygen.



The reaction in which the original ES compound oxidizes some other molecule than hydrogen peroxide has essentially the same mechanism. Alcohol for example can act as a hydrogen donor, reducing the hydrogen peroxide of the ES complex, and being itself oxidized to aldehyde.

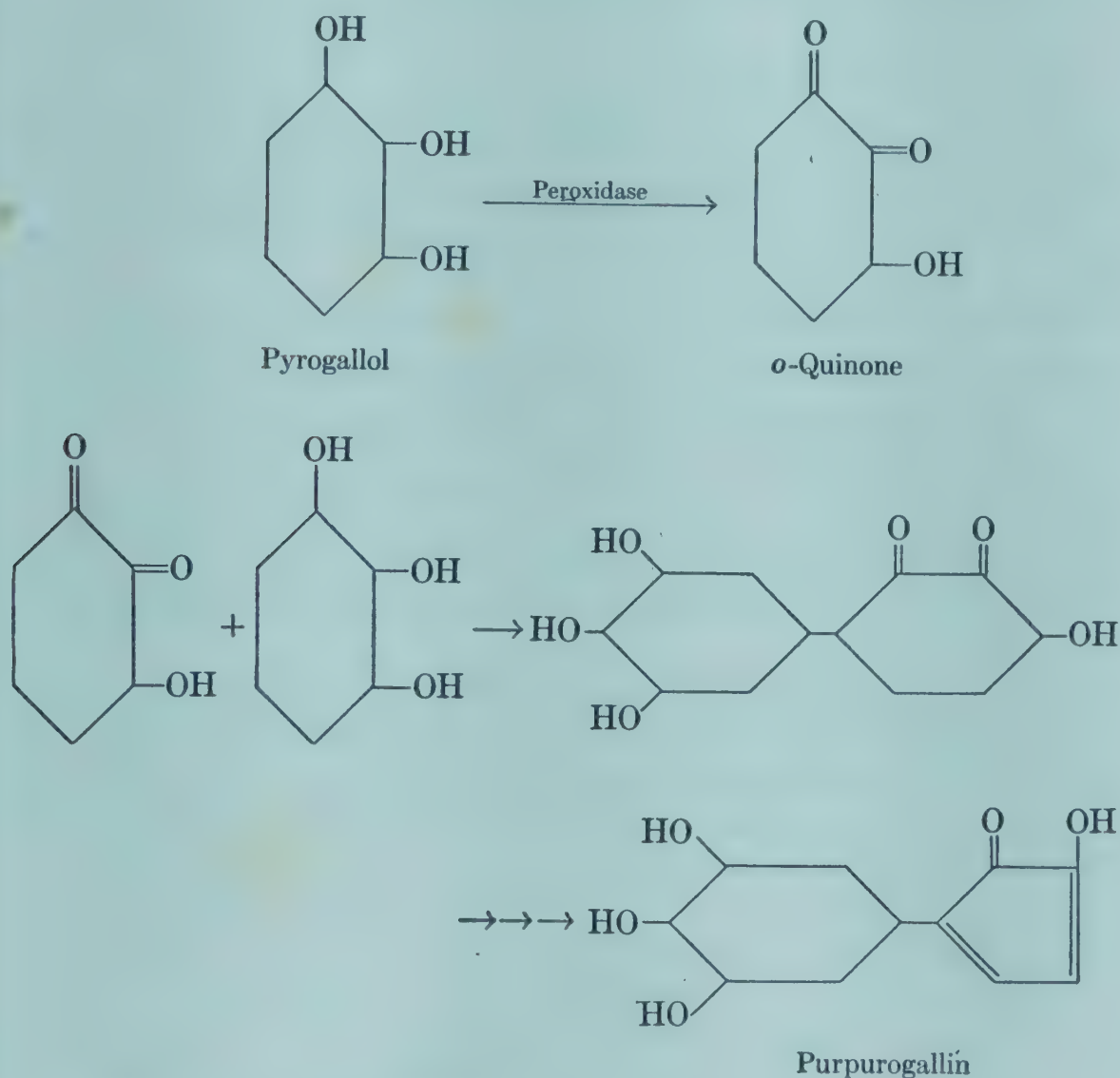


If this second type of reaction catalyzed by catalase has biological significance it is another example of the use of coupled reactions. Peroxide formed perhaps in oxidation by way of a flavin enzyme would thus be put to use by the cell in bringing about enzymic oxidation of some metabolite.

PEROXIDASE: AN IRON PYRROLE ENZYME

Peroxidase is an enzyme which is particularly characteristic of plants and bacteria, though it has been found in some animal tissues also. Horseradish root is commonly used as a source of the enzyme which proves to contain heme as prosthetic group. It catalyzes the oxidation by hydrogen peroxide of various specific substrates including phenols, several amino acids, and some aromatic amines. The substance which is generally used to test for the presence of the enzyme is the trihydric phenol pyrogallol which is transformed catalytically into the orange yellow deriva-

tive, purpurogallin. The initial reaction is an oxidation of the phenol to a quinone, and this substance then condenses with a molecule of the unaltered phenol. After several further transformations the colored purpurogallin results, and this can then be extracted with ether and its amount determined colorimetrically.



When this reaction is used as a test for the activity of an enzyme solution the activity is reported in terms of the purpurogallin number (*Purpurogallin Zahl*, P.Z.), defined as the milligrams of purpurogallin formed per milligram of dried enzyme acting under definitely prescribed conditions. The possible significance of peroxidase was greatly enhanced by the recent discovery that it catalyzes also the oxidation of ferrocytochrome *c* by peroxide.

Peroxidase like catalase is an iron porphyrin protein in which the iron remains in the ferric state during catalytic activity. The enzyme is believed to act as does catalase by first forming an ES compound with the peroxide, which then accepts hydrogen from such a donor as a phenol

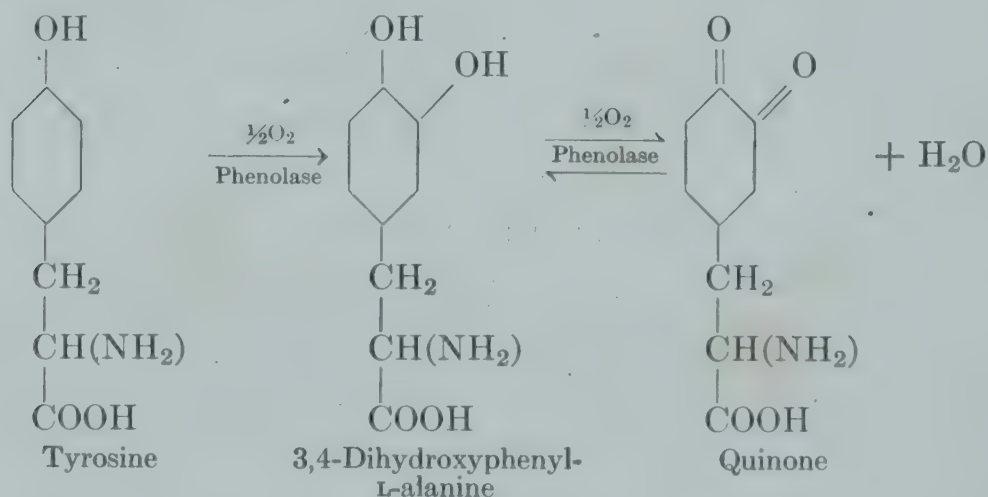
or amine. Thus the function of the peroxidases would appear to be complementary to that of catalase. Each enzyme has its own specific hydrogen donors which can reduce peroxide in the presence of the enzyme protein and its prosthetic group. In addition, catalase can if no such donors are available simply destroy the peroxide and thus put at the disposal of the cell a small amount of added molecular oxygen.

THE COPPER PROTEIN ENZYMES

Just as the iron pyrrole compounds have found a use both in hemoglobins and as oxidative coenzymes, so copper compounds occur in nature not only as respiratory pigments in the hemocyanins, but also as oxidizing enzymes. Such for example are the three copper-containing enzymes, phenolase, laccase, and ascorbic acid oxidase.

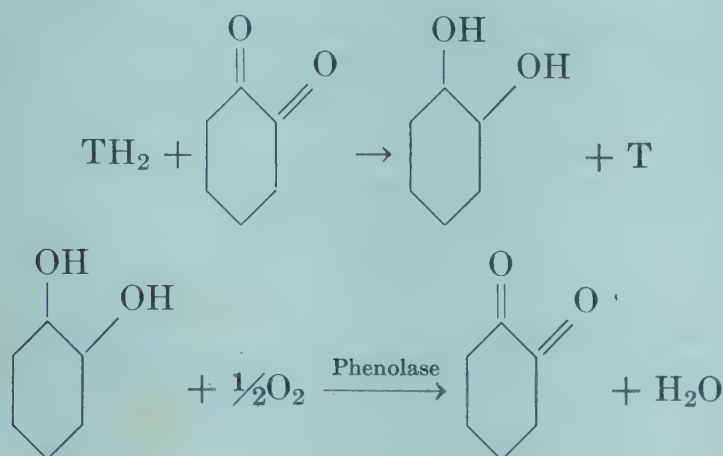
Phenolase. Various plant tissues yield an enzyme which catalyzes the oxidation by molecular oxygen of a number of mono- and di-phenols. The enzyme is a copper protein which undergoes reversible oxidation and reduction of the copper in the course of its catalytic activity. As obtained from different sources it has been known as tyrosinase, polyphenol-oxidase, and phenolase. The latter name is probably preferable, since it indicates that the enzyme has a broad specificity, requiring only that the substrate be either a monophenol or a polyphenol with the second hydroxyl group ortho to the first.

When monophenols are acted upon by phenolase, the first step is addition of oxygen to form an ortho diphenol. This compound is then oxidized to the corresponding quinone. With tyrosine as substrate this quinone may undergo further transformations leading to formation of the dark pigment known as melanin.



As shown in the equation, the oxidation of the diphenol is reversible, and there is some evidence that in the presence of reducing agents the quinone can act as an intermediate hydrogen carrier, transferring hydrogen

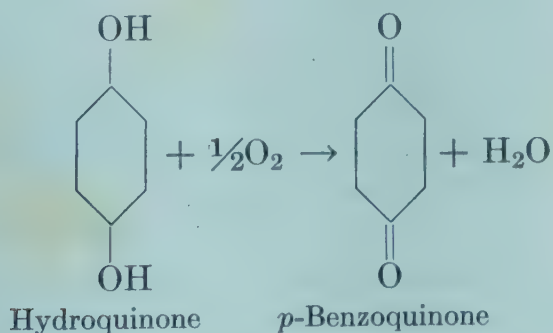
from a metabolite to molecular oxygen. When such a system acts in tissues it is said that the enzyme is acting as a *terminal oxidase*.



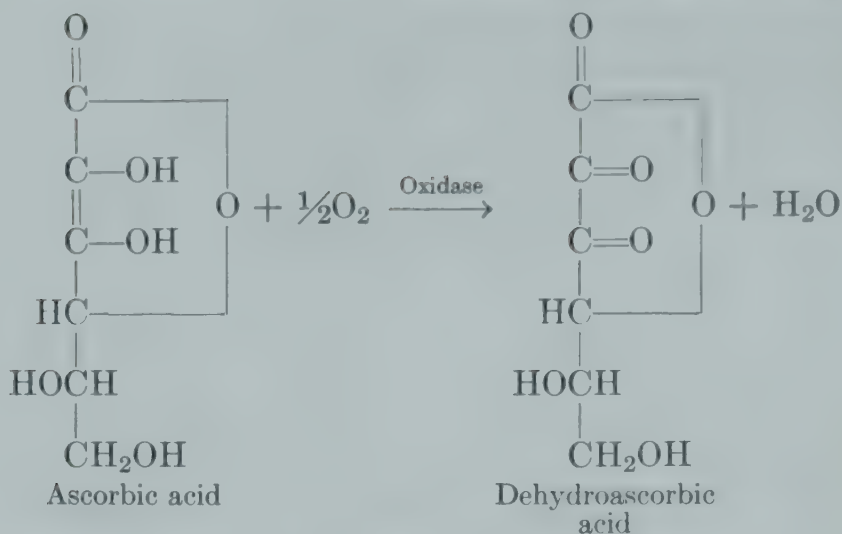
It is believed that phenolase may play a significant part in plant respiration by thus activating oxygen to react with hydrogen, though there is at present no information on the nature of the reducing system designated in the formulation as "TH₂."

Under conditions in which the quinone formed by tyrosine is not exposed to reducing compounds it may be oxidized to form the black melanin pigments which occur in skin and hair and in the colored tumors known as melanomas. A phenolase which has been isolated from some animal tissues has properties which are apparently the same as those of the plant phenolase. This enzyme may therefore be responsible for the initial oxidation of tyrosine and related compounds in animal cells.

Laccase. The copper protein *laccase* is also a phenolase, but one which brings about the oxidation of para- and not of ortho-phenols. It is obtained from the latex of an Indo-Chinese lacquer tree where it acts upon substances present in the latex to form the black resinous lac. Like the other phenolases, laccase catalyzes formation of a quinone from a diphenol.



Ascorbic Acid Oxidase. A third copper protein is the enzyme *ascorbic acid oxidase* found in a wide variety of different plants. It catalyzes the oxidation by molecular oxygen of ascorbic acid, forming dehydroascorbic acid.



Since dehydroascorbic acid is readily reduced it can act as quinone does to transport hydrogen from a metabolite to oxygen. It has been found for example that addition of lactic acid increases the oxygen uptake of respiring barley sap, in which both ascorbic acid and its oxidase occur. This is interpreted to mean that the ascorbic oxidase is acting as a terminal oxidase by means of which hydrogens of lactic acid are transferred to oxygen.



How large a proportion of plant respiration is mediated by this oxidase is not known, but many hydroxy acids occur in plant cells and it may well be that some of them are oxidized by way of ascorbic acid oxidase. It should be noted that in this reaction ascorbic acid, which is the substrate for the oxidase, is also acting as a coenzyme undergoing a reversible cycle of oxidation and reduction.

Formation of Carbon Dioxide

In the course of glycolysis each hexose molecule is transformed into two molecules of pyruvate, and at one step in the sequence two hydrogen atoms are transferred to coenzyme I. If these reactions are taking place under normal aerobic conditions the hydrogens are passed on by way of the cytochromes to oxygen, forming water. When the hydrogens follow this path they are not available to reduce the pyruvate to lactic acid, and the pyruvate is then oxidized completely to carbon dioxide and water.

The elucidation of the series of chemical events by which the three-carbon pyruvate molecule is degraded followed lines which are already familiar. Tissue slices or extracts were tested with different substrates which they were known to oxidize, and attempts were made to identify intermediate products. Whenever a compound seemed to be a likely

intermediate it in turn was tested with the same tissue enzymes. If it proved to be oxidized at least as rapidly as the original substrate from which it was supposed to be derived, its position as an intermediate was strengthened. Gradually it became possible to sketch and then to test a possible sequence of reactions in which the various substrates and their intermediate products could be fitted into a reasonable pattern. From such studies as these there has evolved the theory that formation of carbon dioxide from pyruvate takes place in the course of a cyclical process of a type now familiar. This particular group of reactions is known as the *citric acid cycle*, or the *Krebs cycle*.

In the course of the work on carbohydrate metabolism it had been recognized that most cells can bring about the oxidation of a number of organic acids all of which are structurally related in some degree. It was found furthermore that the enzymes needed for the interconversion of several of these compounds were also present in living tissues. These are the acids which were eventually found to be essential to the oxidation of pyruvate, and which are listed in Table 14-III.

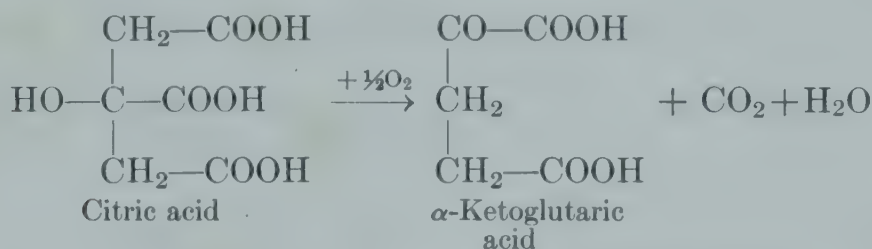
TABLE 14-III. ACIDS RAPIDLY OXIDIZED BY MUSCLE

Citric acid	$ \begin{array}{c} \text{CH}_2\text{—COOH} \\ \\ \text{HO—C—COOH} \\ \\ \text{CH}_2\text{—COOH} \end{array} $
<i>cis</i> -Aconitic acid	$ \begin{array}{c} \text{CH—COOH} \\ \\ \text{C—COOH} \\ \\ \text{CH}_2\text{—COOH} \end{array} $
Isocitric acid	$ \begin{array}{c} \text{CHOH—COOH} \\ \\ \text{CH—COOH} \\ \\ \text{CH}_2\text{—COOH} \end{array} $
α -Ketoglutaric acid	$ \begin{array}{c} \text{CO—COOH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2\text{—COOH} \end{array} $
Succinic acid	$ \begin{array}{c} \text{CH}_2\text{—COOH} \\ \\ \text{CH}_2\text{—COOH} \end{array} $
Fumaric acid	$ \begin{array}{c} \text{CH—COOH} \\ \\ \text{HOOC—CH} \end{array} $
Malic acid	$ \begin{array}{c} \text{CHOH—COOH} \\ \\ \text{CH}_2\text{—COOH} \end{array} $
Oxalacetic acid	$ \begin{array}{c} \text{CO—COOH} \\ \\ \text{CH}_2\text{—COOH} \end{array} $
Pyruvic acid	$ \text{CH}_3\text{—CO—COOH} $

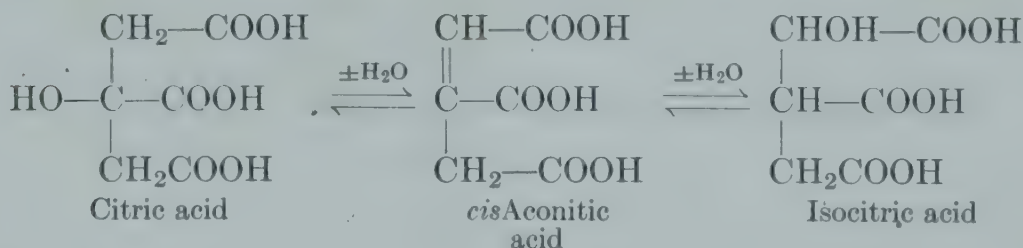
REACTIONS OF THE INDIVIDUAL ACIDS OF THE CITRIC ACID CYCLE

It is obvious from a glance at the table that the acids listed can be derived from each other by simple reactions of the types known to take place in living tissue, such as hydrogenation, removal or addition of the elements of water, and decarboxylation. The enzymes which catalyze these reactions have been found in nearly all tissues which have been examined, whether plant, bacterial, or animal. This points to a very wide applicability for the cycle in which these enzymes are involved. The reactions which are pertinent to our present purpose are outlined in the following sections.

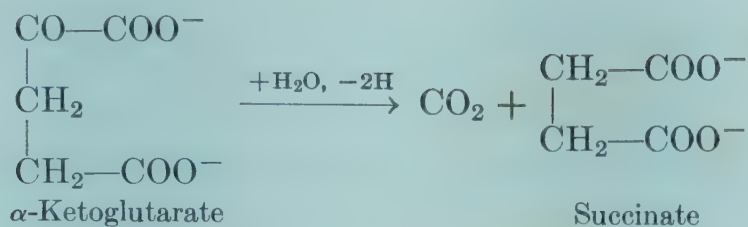
Citrate. 1. Oxidation of citric acid, catalyzed by a dehydrogenase from cucumber seeds, was shown to use only one atom of oxygen per molecule of acid, and to give rise to one molecule of carbon dioxide. It was later shown that the other oxidation product is α -ketoglutarate and that the reaction is catalyzed by animal tissue also. Obviously this reaction involves more than the single step here indicated.



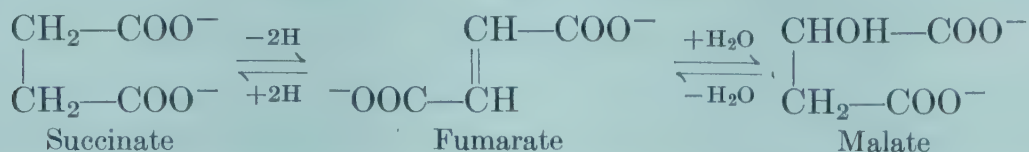
2. The other important fact about citric acid is that many tissues contain an enzyme *aconitase* which brings about an equilibrium involving the three tricarboxylic acids, citric acid, isocitric acid, and *cis*aconitic acid.



α -Ketoglutarate. Decarboxylation is an enzymic reaction which is characteristic of α -ketonic acids and it therefore might be expected that α -ketoglutarate would be converted oxidatively into succinic acid. In 1937 Krebs and Johnson succeeded in demonstrating this transformation in muscle tissue. In order to isolate the succinic acid it is necessary to carry out the reaction in the presence of the competitive inhibitor malonic acid. Under these conditions the further enzymic oxidation of succinic acid is prevented and this acid therefore accumulates.

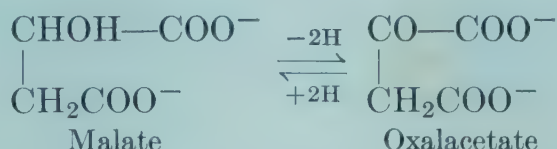


Fumarate. 1. Fumarate, succinate, and malate have long been known to be interconvertible in biological systems. Succinic dehydrogenase

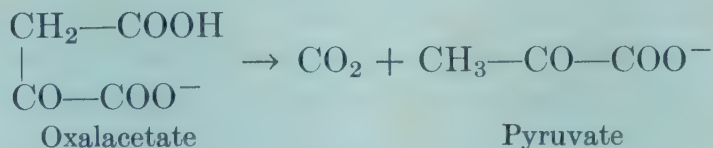


catalyzes the reversible formation of fumarate from succinate; the *fumarase* which brings about the reversible transformation of fumarate to malate is widely distributed in the tissues of animals, plants and bacteria.

2. Oxidation of fumarate or malate by tissue preparations leads to formation of oxalacetate. This is believed to come about by dehydrogenation of malate in both cases, the fumarate first forming malate by addition of water.

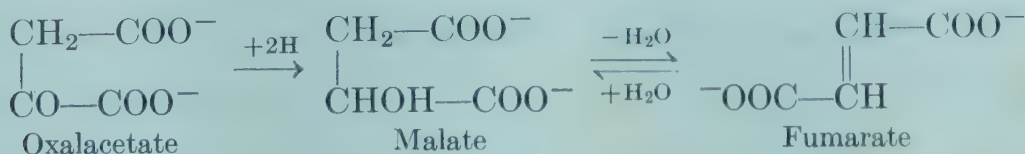


Oxalacetate. 1. Decarboxylation of oxalacetate takes place so readily in water solution that it is scarcely necessary to search for a catalytic agent.



However, such an enzyme has been found in bacterial cells and in liver tissue. Magnesium or manganese ions are necessary parts of the enzyme systems involved.

2. Reduction of oxalacetate can be brought about by enzymes present in muscle and in bacteria. The reaction yields a mixture of malate and fumarate which is in some tissues further reduced to succinate.



3. The amount of oxalacetate which disappears from muscle suspension is always greater than can be accounted for by decarboxylation and re-

duction. Search for other products indicated some formation of the five-carbon α -ketoglutarate and the six-carbon citrate. The mechanism of these syntheses will be considered later.

Pyruvate. Pyruvate is rapidly oxidized by many different varieties of living tissue. When this oxidation is measured in minced muscle, and in the absence of inhibitors, it is quantitative, two and a half moles of oxygen being used and three moles of carbon dioxide formed, per mole of pyruvate disappearing. In the presence of malonate acting as an enzyme

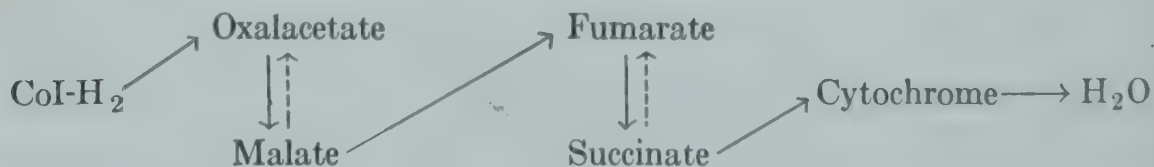


inhibitor, the pyruvate disappears, but less oxygen is used and succinate accumulates. This suggests that the four-carbon succinate is an intermediate in the oxidation of the three-carbon pyruvate.

Furthermore, when a mixture of pyruvate and fumarate is metabolized by muscle suspension, significant amounts of both citrate and α -ketoglutarate form. Here again is the transformation of a short carbon chain into a longer one. These two cases plus that of oxalacetate in (3) above, point to the conclusion that biological oxidation is not a simple series of catabolic steps, but must include some building up of carbon chains. A number of different schemes have been proposed to account for the facts and these have gradually merged into the modern theory originated in 1937 by Krebs and Johnson.

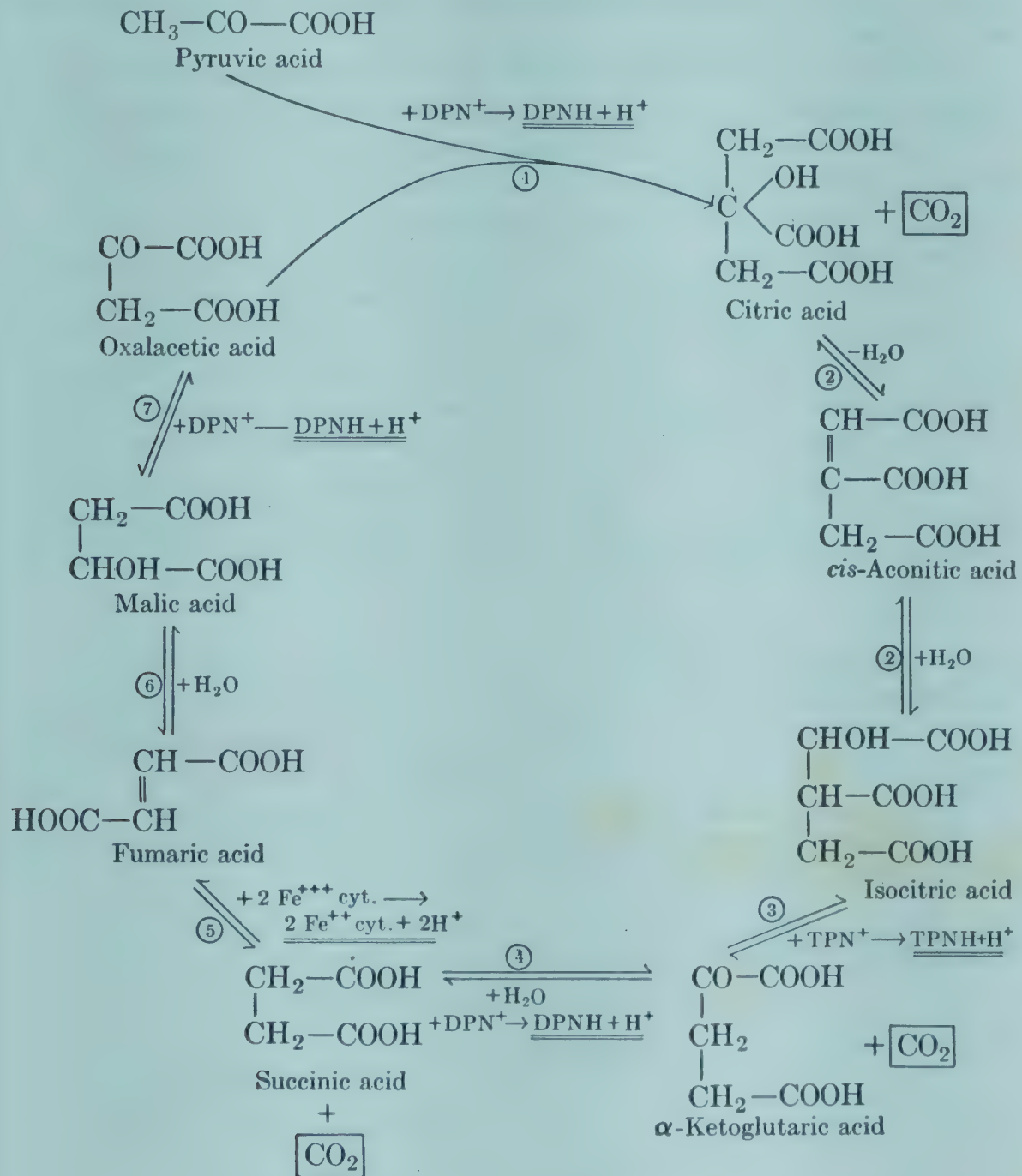
THE CITRIC ACID CYCLE

Understanding of the metabolic importance of the dicarboxylic acids began about 1936. In that year Szent-Györgyi reported that addition of succinate, or of any of the four-carbon acids to which it gives rise on oxidation, acted catalytically upon the oxygen uptake of muscle. That is, the increased oxygen uptake was greater by several fold than would have been found had the added substances acted simply as additional substrates. This suggested that they must be acting in some sort of cyclic mechanism. Szent-Györgyi's theory was that they acted to bridge the gap between the dehydrogenases and the cytochrome system, as indicated in the following scheme. Hydrogen from a reduced coenzyme would follow the lines of the solid arrows, being finally donated to a cytochrome by succinate, catalyzed by succinic dehydrogenase.



A year later Krebs and Johnson reported new observations which led them to postulate a more inclusive cycle of polycarboxylic acids.

1. Citrate was shown to exert the same catalytic effect on respiration of pigeon breast muscle as did the four-carbon acids.
2. Fumarate or oxalacetate added to respiring tissue were found to give rise to succinate by *oxidation*, that is, with a simultaneous uptake of oxy-



THE CITRIC ACID CYCLE

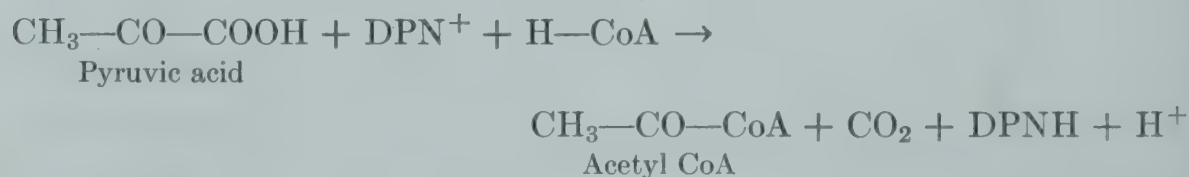
Figure 14.7. The series of reactions by which pyruvic acid is degraded to carbon dioxide and water.

gen. If the experiment were conducted anaerobically almost no succinate appeared. This is exactly contrary to what might have been expected. Formation of succinate from fumarate by *reduction* would of course have been familiar enough, yet did not take place. Furthermore, this ordinary

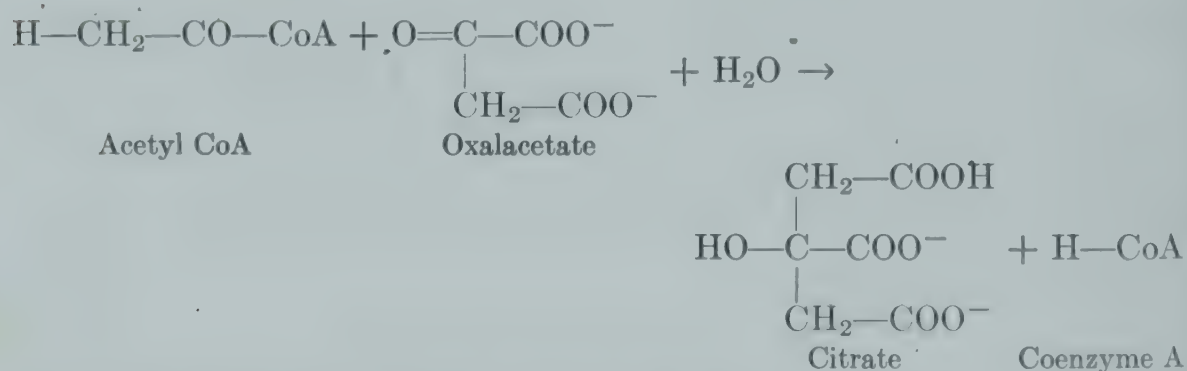
reductive formation of succinate from fumarate is completely inhibited by malonate, yet when fumarate was added to respiring tissue poisoned with malonate, succinate still formed, with a concomitant uptake of oxygen. This could only mean that oxidative removal of the four-carbon fumaric acid led in some undefined way to synthesis of the four-carbon succinic acid.

The series of cyclic changes which Krebs and Johnson postulated to account for all the facts is indicated in Figure 14.7. The operation of the cycle may be said to "begin" when pyruvate, formed anaerobically from glucose or glycogen, condenses with oxalacetate to yield citric acid, and to "end" when oxalacetate is again reconstituted.

Reactions of the Cycle. 1. For many years there was much uncertainty about the course of the first step. It was not known whether the pyruvate lost carbon dioxide and then condensed with oxalacetate to form one of the six-carbon acids, or whether a "procitric" acid containing seven carbons was first formed and then decarboxylated. Quite recently Ochoa has shown that the first reaction is an oxidative decarboxylation in which coenzyme I acts as hydrogen acceptor. The reaction takes place only in the presence of coenzyme A and involves formation of the acetyl derivative to which reference has already been made several times. This may be formulated



It is then the acetylated coenzyme which reacts with oxalacetate and water to form citric acid and regenerate the coenzyme.

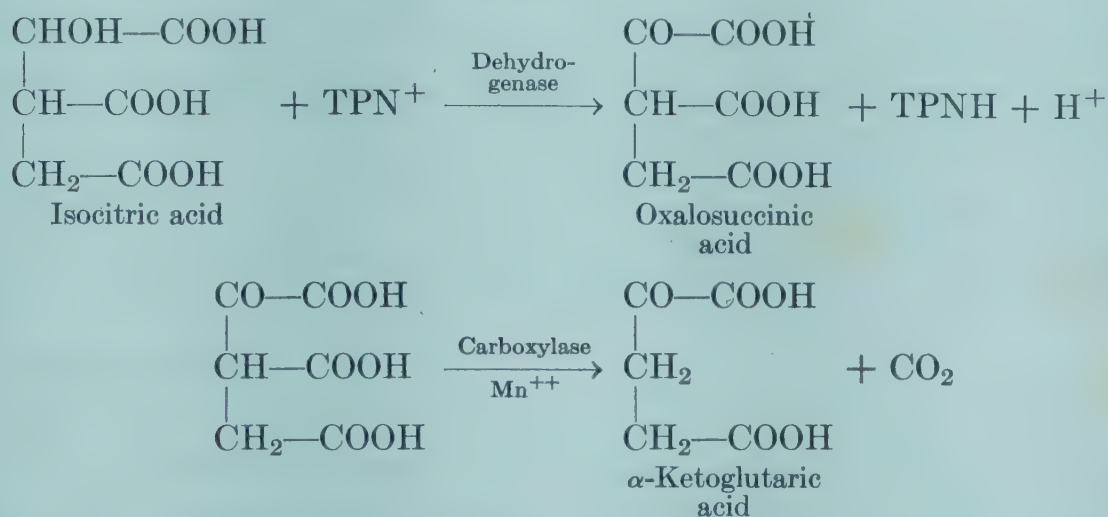


Thus as a result of these first steps in the cycle, citric acid is formed and two hydrogen atoms are started on their oxidative path toward cytochrome.

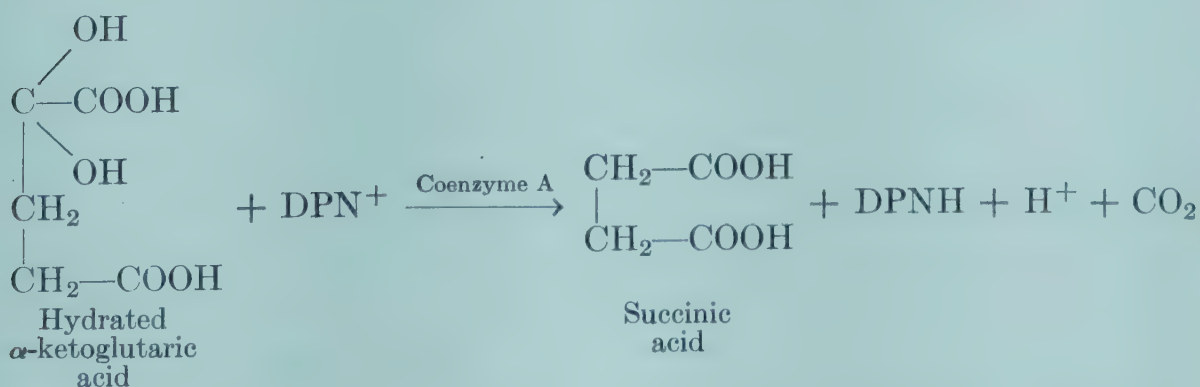
2. Under the influence of *aconitase* the three tricarboxylic acids form an equilibrium mixture. This equilibration reaction when it takes place in

tissue is driven continually toward formation of isocitric acid by the oxidative removal of this latter compound by the next reaction of the cycle.

3. The third reaction of the cycle takes place in two steps. In the first, isocitric dehydrogenase catalyzes the transfer of a second pair of hydrogens which are accepted by coenzyme II. This step would yield oxalosuccinate by removal of the hydrogens from the secondary alcohol group. But since there is a simultaneous decarboxylation the actual product is α -ketoglutarate. This second step is mediated by a specific carboxylase which requires the presence of manganese ions.

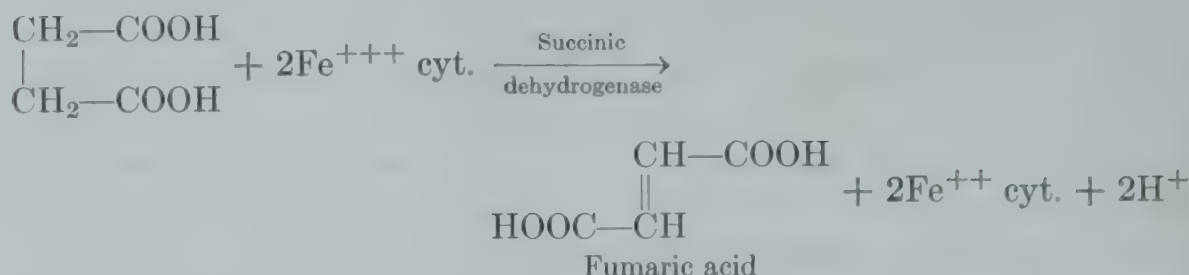


4. This is again an oxidative decarboxylation in which a third pair of hydrogen atoms is donated, this time to coenzyme I, while a third molecule of carbon dioxide is split out. The latter reaction is mediated by a specific carboxylase which requires as coenzyme the same diphosphothiamine or "cocarboxylase" which acts in decarboxylation of pyruvic acid in the fermentation sequence. It has very recently been found that the oxidation also requires the presence of coenzyme A, but the intermediate stages have not yet been completely elucidated. The over-all reaction may be formulated as follows, writing the keto group of the acid in the hydrated form to account for the transfer of hydrogens to coenzyme I.

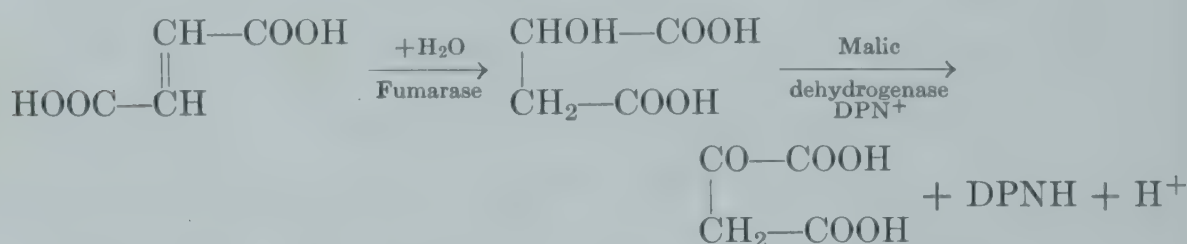


5, 6, and 7. In these reactions the four-carbon acids undergo the transformations indicated in the chart. Succinic dehydrogenase mediates the

transfer of the first pair of hydrogens from succinate to the cytochrome system.



Under the influence of fumarase water is then added to fumaric acid to form malic acid, and this compound is in turn dehydrogenated through the catalytic action of malic dehydrogenase.



Thus in the course of one complete cycle ten hydrogen atoms have been set upon an oxidative pathway which will lead ultimately to their uniting with five atoms of oxygen, via cytochrome oxidase. Three molecules of carbon dioxide have also been set free. These quantities are exactly those required by the stoichiometric equation for complete oxidation of pyruvic acid. Furthermore they correspond to the experimentally determined uptake of oxygen and output of carbon dioxide per molecule of pyruvate oxidized completely in minced muscle.



It is now generally believed that in many different kinds of tissue the oxidation of carbohydrate takes place through the citric acid cycle. The necessary enzymes are widely distributed in animal tissues, in some microorganisms and in many plant cells. Many of the acids involved are so common in plants as to be known as "plant acids," and there is direct evidence that in some plant tissues oxidation of pyruvate leads to succinate formation. If the citric acid cycle has this general importance, it means that the glycolytic reactions are not, as was once believed, merely an inefficient way of obtaining energy anaerobically. They constitute rather an essential preparation of carbohydrate molecules for oxidation, by degrading them to the pyruvate stage. This substance then normally enters the oxidative Krebs cycle. It is only when oxygen is lacking or limited in amount that pyruvate is reduced to lactic acid, as in muscle, or decarboxylated to acetaldehyde as in alcoholic fermentation.

OXIDATION OF FATTY ACIDS

The Krebs cycle has proved to be of even more general significance than as a vehicle for carbohydrate oxidation. It will be recalled that the fatty acids are metabolized in such a way that two-carbon fragments are broken off in sequence in the form of acetyl coenzyme A. Work with isotopically labeled fatty acids has shown that their carbons are oxidatively incorporated into the acids of the citric acid cycle, as one would expect if they give rise to the same acetylated coenzyme as is formed by pyruvate. Thus the two-carbon fragments from the fatty acids are normally funneled into the citric acid sequence, through which they are degraded to carbon dioxide and water. It is perhaps when this process cannot take care of the acetyl CoA complexes as fast as they are formed that some of them recondense to form acetoacetate. Presumably the oxidative use of this substance by the peripheral tissues involves the re-formation of two molecules of the acetylated coenzyme A which then condense as usual with oxalacetate to enter the citric acid cycle.

OXIDATION OF AMINO ACIDS

Many amino acids are also oxidized by way of the Krebs cycle. Three amino acids for example are known to give rise by deamination to acids which are intermediates of the cycle. Once these products have entered the metabolic pool they are of course indistinguishable from similar molecules from other sources.

Glutamate \rightarrow α -Ketoglutarate

Aspartate \rightarrow Oxalacetate

Alanine \rightarrow Pyruvate

Several other amino acids are known to be transformed in the animal body to glutamate which then enters the cycle by deamination. These acids are histidine, ornithine, proline, hydroxyproline, and arginine. In the course of oxidation phenylalanine or tyrosine give rise to fumarate, by way of which their further oxidation may be carried out through the cycle. Finally there are the ketogenic amino acids, leucine, phenylalanine, and tyrosine, which give rise under some circumstances to acetoacetate. They may therefore enter the cycle as do the fatty acids by way of acetyl CoA. Thus a substantial number of the amino acids are known to be oxidized through the Krebs cycle and it seems likely that others will also prove to use that mechanism after suitable transformation.

Organized Respiratory Activity

Until quite recently most enzyme studies were directed toward the isolation, purification, and characterization of individual enzymes. This

phase has now led into a later one in which two separate lines of work converge to throw light on the organization of oxidative catalysts in the living cell.

MITOCHONDRIA AND THE CYCLOPHORASE SYSTEM

Under some experimental conditions pyruvate oxidation is a one-step reaction, yielding acetate and carbon dioxide. Searching for a source of the oxidase involved in this transformation, Green tested homogenates of rabbit liver and kidney. These preparations in the presence of pyruvate give rise to a very rapid uptake of oxygen, in the course of which the pyruvate is completely oxidized to carbon dioxide and water by way of the citric acid cycle. Attempts to fractionate the enzyme mixture failed completely. When the homogenate was centrifuged all the enzymes were found in that particulate fraction which sedimented in a low centrifugal field. Concluding finally that this association of enzymes was not simply a chance mixture but an organized structural unit, Green and his group gave the name *cyclophorase* to the enzyme system associated with the particles.

At about the same time Lehninger and Kennedy at the University of Chicago and Schneider and Potter at the University of Wisconsin were using differential centrifugation to separate into fractions the cell contents extracted after rupturing the cell membranes. Table 14-IV indicates the chief particulate fractions obtained in the early experiments.

TABLE 14-IV. FRACTIONATION OF PARTICULATE ELEMENTS OF RAT LIVER ^a

Fraction	Diameter (μ)	Sedimentation	
		Time (min.)	Acceleration (g.)
Nuclei	50-100	10	600
Mitochondria	1-3	20	24,000
Microsomes	0.06-0.15	120	41,000

^a Adapted from E. L. Lehninger, in *Enzymes and Enzyme Systems*, John T. Edsall, ed., Harvard University, Cambridge, Mass., 1951.

Study of the separated particles revealed that the mitochondria were enormously more active catalysts of oxidation than either of the other two. Indeed the complete battery of enzymes required for the citric acid cycle was concentrated in that fraction. This confirmed the hypothesis put forward by Green that the cyclophorase system in the cell represents an integrated structural unit. It also threw the first light on the function of those tiny rods and filaments, the mitochondria, which dot the protoplasm. Of the relationship between structural unit and enzyme complex Green has written: ". . . few would be prepared to contest that the cyclo-

phorase system of enzymes represents a preëxisting organized bit of the cells' enzymatic machinery . . ."

Mitochondrial preparations of rat liver have proved capable of oxidizing rapidly (a) the members of the citric acid cycle, (b) fatty acids with some formation of acetoacetate, and (c) several amino acids. The connecting link between these diverse activities is of course the citric acid cycle through which all the oxidations ultimately funnel. Green has also shown that in the course of these oxidations, inorganic phosphate is incorporated into the cyclophorase gel as a very labile phosphate ester. This fits in with the hypothesis that a major metabolic pathway is that by which chemical energy freed in oxidation is stored as high energy phosphate bonds. At present too little is known of this reaction at the mitochondrial level to attempt a chemical formulation. But even now it is abundantly clear that the mitochondrial bodies are extraordinarily complex and complete structural units, containing the enzymes and coenzymes required, not only for operation of the citric acid cycle, but for a wide variety of ancillary reactions. Work with a mitochondrial preparation from mung bean indicates that in plant cells also these particles carry a battery of oxidative enzymes entirely comparable with those in animal tissues.

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Study Questions

1. On what experimental evidence did Warburg base his theory of oxygen activation?
2. What was Wieland's theory, and on what experimental evidence did it rest?
3. What is the Thunberg technique? How was it used to prove the presence of dehydrogenases in tissue? How may it be adapted to measure the concentration of dehydrogenases?
4. What is a coenzyme? How is its existence discovered?
5. What is a pyridinoprotein enzyme? To what part of the enzyme is its specificity due?
6. What is the actual chemical reaction which takes place when a pyridine enzyme acts as catalyst?
7. What is the structure of coenzyme I? How does it differ from coenzyme II?
8. What does DPN stand for? TPN? FAD? FMN?
9. What simple experimental evidence indicates that cytochrome can be reversibly oxidized and reduced?
10. Which part of the catalytic action on the cytochromes is inhibited by narcotics? which by cyanide?
11. From what substances can cytochrome *c* accept hydrogen? To what substance can the cytochromes donate hydrogen?
12. What type of compound is cytochrome oxidase? To what group of compounds does cytochrome reductase belong?
13. What is the structure of the flavin coenzymes? By what chemical reaction do they function in hydrogen transport?

14. What type of enzyme is catalase? What is known of the mechanism of its catalysis?
15. What is the peroxidase type of catalysis?
16. What is known of the mechanism of catalysis by phenolase?
17. What is meant by a "terminal oxidase"? Which is the chief one in animal tissues? Which may serve this function in plants?
18. List the acids of the citric acid cycle with their formulas.
19. What is the first step in the oxidation of pyruvate by way of the citric acid cycle?
20. What are the products of one complete citric acid cycle?
21. Which coenzymes accept hydrogens in the reactions of the citric acid cycle?
22. By what type of reaction is carbon dioxide formed in the citric acid cycle?
23. What is the relation of fatty acid oxidation to the citric acid cycle?
24. How is protein oxidation related to the citric acid cycle?
25. Where is the oxidation mechanism believed to be localized in the intact cell?

Biosynthetic Mechanisms

Wherever [the reader] finds that I have ventur'd at any small conjectures, at the causes of the things that I have observed, I beseech him to look upon them only as doubtful Problems, and uncertain gheses, and not as unquestionable Conclusions, or matters of unconfutable Science; I have produced nothing here, with intent to bind his understanding to an implicit consent. . . .

ROBERT HOOKE: *Micrographia* (1665)

In the early days of biochemistry a distinction was made between catabolism, or the breakdown of the foodstuffs, and anabolism, or the synthesis of cell substances. It must already be clear that no such definite classification is possible. For example, in the course of degrading pyruvate to carbon dioxide and water, citrate is synthesized; the catabolic end product urea is formed in a process which involves several complex synthetic reactions; a deaminated amino acid seems clearly embarked upon a catabolic path, yet either its nitrogen or its carbon chain may in a very short time appear in tissue proteins. Clearly the interplay of degradation and synthesis is complex and labile.

Biosynthesis in Heterotrophic Organisms

As far as we now know the fundamental units used by the cell in the synthesis of carbon compounds are carbon dioxide and water and the small one- and two-carbon fragments which have been called "active formate" and "active acetate." We have seen that the products of fatty acid metabolism can best be explained if we assume that two carbons are split off at a time, and that some of these small units are used again in synthesis. Thus, acetoacetate is believed to arise from condensation of two such molecules. But this type of condensation is not a characteristic reaction of the familiar substance acetic acid, hence it has been postulated that the two-carbon compound involved must be something related to acetic acid, but much more reactive, hence called provisionally "active acetate." Similarly the existence of a one-carbon synthetic precursor is indicated by experiments in which the carbon of labeled formate is incorporated by means of cell catalysts in a number of different compounds. It is the purpose of this chapter to consider what is now known of the mechanisms by which living

cells synthesize complex carbon compounds from simple precursors. Since this whole problem is very new, much that is speculative will inevitably be found in the following discussion, and no final conclusions can be drawn. The best that can be offered is a survey of work in progress, and some indication of the direction in which contemporary biochemical thought is moving.

PHOSPHATE BOND ENERGIES

In the discussion of anaerobic carbohydrate metabolism it was noted that each complete glycolytic cycle generates two or more molecules of adenosine triphosphate, ATP. By this mechanism part of the chemical energy of the carbohydrates is conserved in the form of high energy bonds.

ATP is one of a small group of phosphorus compounds which are essential to the functioning of all cells. Formation of a glucosidic or ester phosphate linkage requires the expenditure of only 2000–4000 calories per mole. The phosphate bond in ATP on the other hand is of the high energy

TABLE 15-I. COMPOUNDS HAVING ENERGY RICH PHOSPHATE BONDS

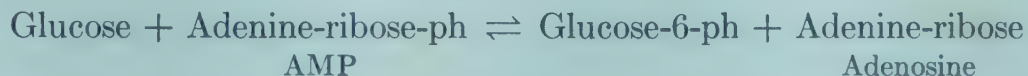
$ \begin{array}{c} \text{NH}_2 \\ \\ \text{C} \\ // \quad \backslash \\ \text{N} \quad \text{C} = \text{N} \\ \quad \backslash \quad / \\ \text{HC} \quad \text{C} = \text{N} \\ \quad \quad \backslash \quad / \\ \quad \quad \text{CH} \end{array} \text{---} \text{C}_5\text{H}_8\text{O}_4 \text{---} \text{P}(\text{OH})(\text{O}) \text{---} \text{O} \sim \text{P}(\text{OH})(\text{O}) \text{---} \text{O} \sim \text{P}(\text{OH})(\text{O}) \text{---} \text{OH} $	Adenosine triphosphate
$ \begin{array}{c} \text{NH} \\ \\ \text{HN} \text{---} \text{C} \text{---} \text{NH} \text{---} (\text{CH}_2)_3 \text{---} \text{CH} \text{---} \text{COOH} \\ \qquad \qquad \qquad \\ \text{PO}_3\text{H}_2 \qquad \qquad \text{NH}_2 \end{array} $	Arginine phosphate
$ \begin{array}{c} \text{CH}_2 = \text{C} \text{---} \text{COOH} \\ \\ \text{O} \sim \text{PO}_3\text{H}_2 \end{array} $	Phosphoenolpyruvic acid
$ \begin{array}{c} \text{CH}_2\text{O} \text{---} \text{PO}_3\text{H}_2 \\ \\ \text{CHOH} \\ \\ \text{C} = \text{O} \\ \\ \text{O} \sim \text{PO}_3\text{H}_2 \end{array} $	1,3-Phosphoglyceric acid
$ \begin{array}{c} \text{NH} \sim \text{PO}_3\text{H}_2 \\ \\ \text{C} = \text{NH} \\ \\ \text{N} \text{---} \text{CH}_3 \\ \\ \text{CH}_2\text{COOH} \end{array} $	Creatine phosphate
$ \begin{array}{c} \text{CH}_3 \text{---} \text{CO} \sim \text{PO}_3\text{H}_2 \\ \\ \text{O} \end{array} $	Acetyl phosphate

type and requires approximately 10,000–12,000 calories per mole. In Table 15-I are listed the phosphate compounds which are known to be energy rich. In the formulas Lipmann's convention has been adopted, by which a high energy phosphate bond is represented as $\sim\text{ph}$ or $\sim\text{PO}_3\text{H}_2$, to distinguish it from such energy poor bonds as those in glycerophosphate or the hexose phosphates. Thus the formula of ATP as written shows that only the two terminal pyrophosphate links are of the high energy type. Arginine phosphate, with its energy rich bond from phosphorus to nitrogen serves in the muscles of many invertebrates the purpose served in the muscles of vertebrates by creatine phosphate.

We have already had examples of the transfer of $\sim\text{ph}$ from one energy rich compound to another. Synthesis of ATP by transfer of a phosphate group from phosphoenolpyruvate to ADP takes place in the course of the glycolytic cycle, and the concentration of creatine phosphate is maintained in muscle by transfer of high energy phosphate from ATP to creatine. In general since these reactions involve no large energy change there is ready interconvertibility on the high energy level.



On the lower level also phosphate can be transferred from one linkage to another, either within the same compound or by reaction between two compounds. Thus the phosphate group in glucose-1-phosphate is readily transferred to carbon 6 or the low energy phosphate of adenosine monophosphate (AMP) to glucose.



Such interchanges as these also involve very little exchange of energy. Figure 15.1, from a paper by Lipmann, summarizes the information about these interrelationships. To raise a phosphate group from the inorganic level requires provision of energy but when an energy rich phosphate group drops to either of the lower levels energy is set free. Any changes within a level take place with only slight energy changes.

In vertebrate muscle the energy which is stored in ATP and in creatine phosphate is used directly or indirectly for the mechanical work of contraction. But ATP has a much more universal function than this. It serves also in ways which are only beginning to be understood as an energy source for many cell functions such as synthesis and bioluminescence. Thus in the normal course of metabolism the inorganic phosphate present in the cell fluids goes through a cycle of changes. It is raised through the intervention of energy of oxidation to the high energy level, and then, releasing this energy for synthetic use, it reverts to the inorganic level again. This cycle is represented schematically in Figure 15.2, taken from

a paper by Lipmann. The metabolic wheel represents the sum of the oxidative reactions by which phosphate is raised from the inorganic level and given the potential which can then be used mechanically or syn-

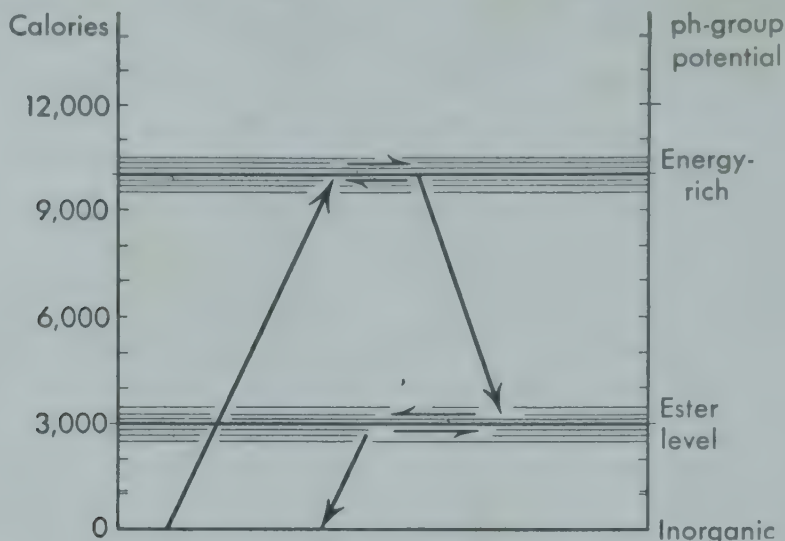


Figure 15.1. Relationship between phosphate bonds at different energy levels. (From F. Lipmann, "Metabolic Generation and Utilization of Phosphate Bond Energy," in *Advances in Enzymology*, 1:126. Copyright 1941, Interscience Publishers, Inc., New York.)

thetically or in other ways. It is with the part of the cycle labeled "utilization" that we are now primarily concerned. It has been found that many of the reactions which are believed to involve active acetate

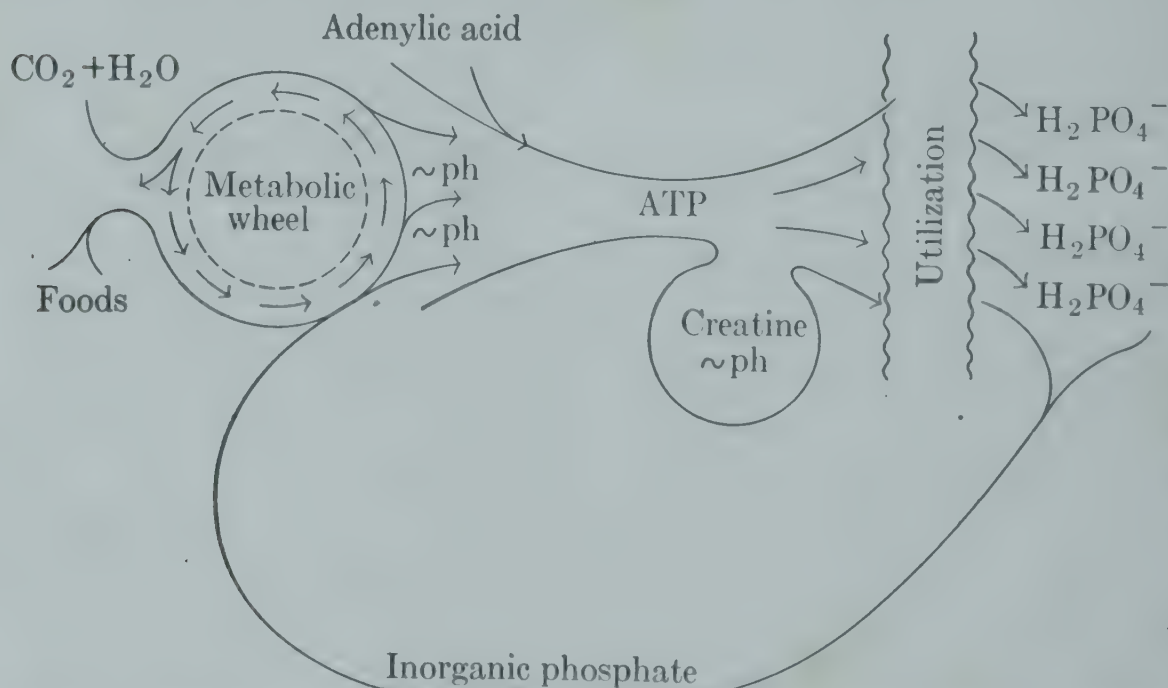
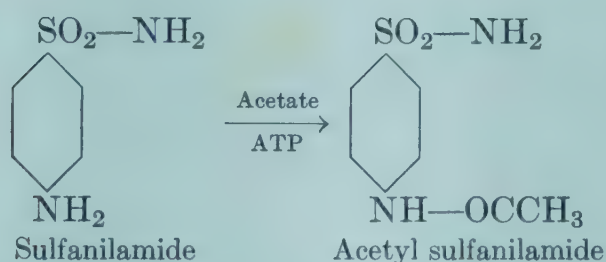
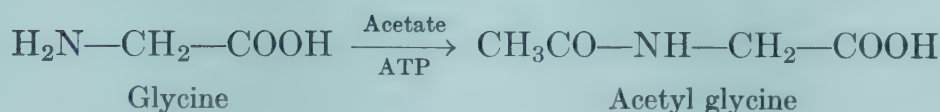
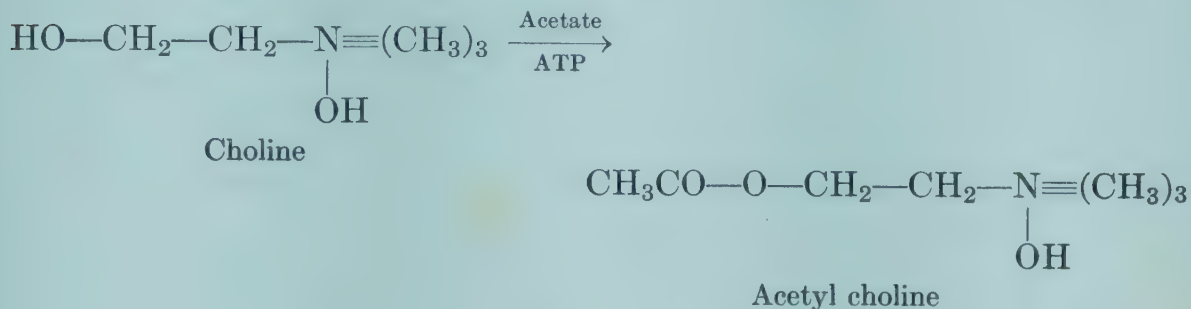


Figure 15.2. The metabolic dynamo is represented as generating high energy phosphate bonds ($\sim\text{ph}$). This is captured in ATP which distributes the energy and returns the inorganic phosphate to the cell, to be caught up again in the metabolic wheel. (After F. Lipmann, "Metabolic Generation and Utilization of Phosphate Bond Energy," in *Advances in Enzymology*, 1:122. Copyright 1941, Interscience Publishers, Inc., New York.)

take place only in the presence of ATP. This has been interpreted as evidence that ATP is the immediate source of the energy which makes the two-carbon unit "active."

ACTIVE ACETATE

There are a few reactions which have proved especially useful in the search for active acetate. There are for example acetylation reactions, catalyzed by bacterial systems as well as by liver enzymes. Acting upon added acetate these enzyme extracts bring about acetylation of choline or of amino acids or of aromatic amines. As noted in the equations these syntheses all require the presence of ATP.



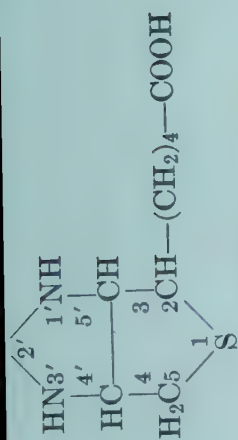
The formation of acetyl choline by cell enzymes is of special interest because of the importance of this substance in the transmission of nerve impulses. The acetylation of sulfanilamide is believed to be a detoxication mechanism, as it is in the form of the acetylated derivative that the drug is excreted by man. The fact that all these reactions require the presence of ATP made it seem likely that an active two-carbon unit was formed at some point in the acetylations.

Another very similar reaction which is widely used to determine whether or not a given precursor yields active acetate is the synthesis of acetoacetate. In the presence of suitable cell enzymes and of ATP, labeled acetate gives rise to labeled acetoacetate. Thus clarification of the mechanism of this reaction would also be likely to uncover any intermediate having the properties of active acetate.

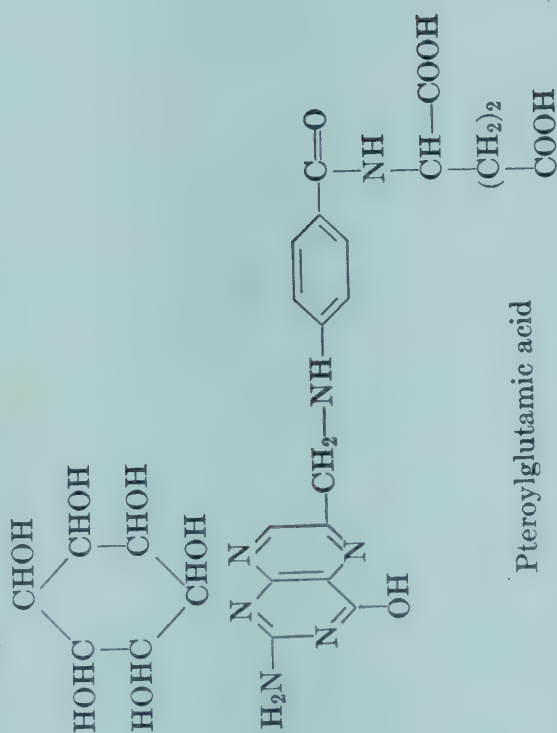
Acetyl Coenzyme A. Working with bacterial enzyme systems which bring about various acetylation reactions, Lipmann discovered that on dialysis,

TABLE 15-II. THE CHIEF MEMBERS OF THE GROUP OF B VITAMINS

Name	Formula	Known Chemical Function
B ₁ or Thiamine		Thiamine pyrophosphate is cocarboxylase
B ₂ or Riboflavin		As monophosphate or as flavinadenine dinucleotide is part of at least 2 oxidizing coenzymes
Nicotinic acid		As the amide in coenzymes I and II
B ₆ or Pyridoxine		As phosphate of the closely related aldehyde in the coenzyme for transamination and decarboxylation
Pantothenic acid	<p>5-Hydroxy-6-methyl-3,4-pyridinedimethanol</p>	In coenzyme A

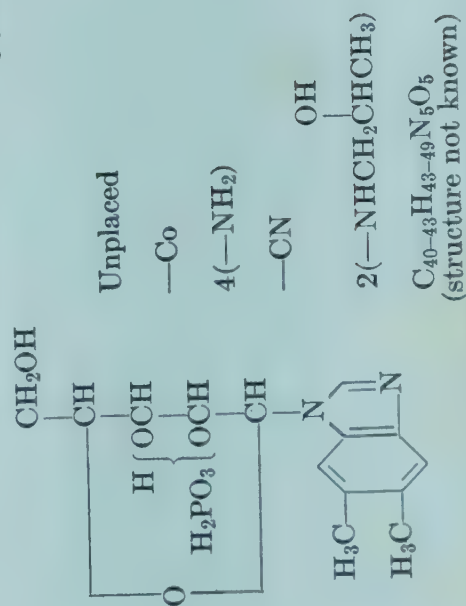


Inositol



Folic acid

Pteroylglutamic acid

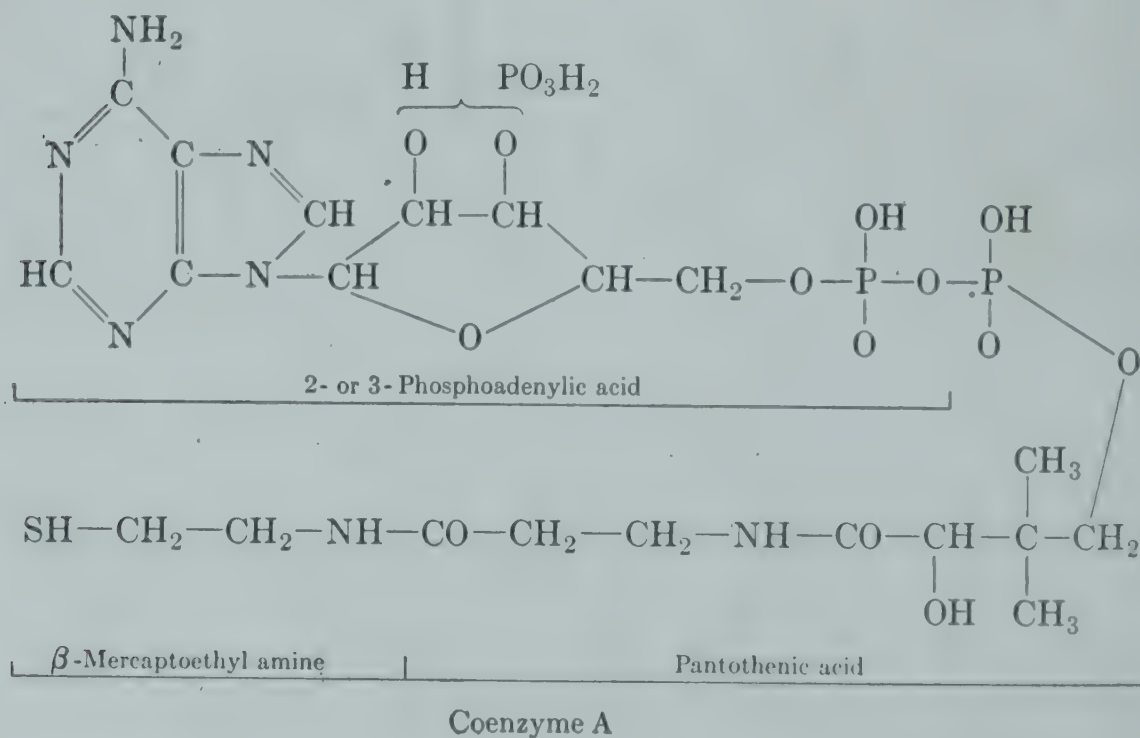


B₁₂ or Cyanocobalamin

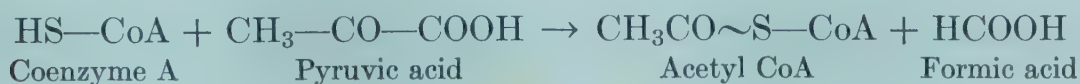
or even on aging, they lost some essential factor. This proved to be a heat-stable substance which, as a factor involved in acetylations, was named Coenzyme A.

Chemistry of Coenzyme A: One of the most interesting developments of recent years has been the recognition of the specific functions in cell metabolism of the various members of that heterogeneous group of compounds known collectively as the B vitamins. Beginning in the early days of vitamin study as a supposedly single substance, "water-soluble B," the group is now known to include at least nine members which are listed in Table 15-II. We have already noted the use in oxidizing coenzymes of nicotinic acid and of vitamin B₂ and the presence of pyridoxine or B₆ in the coenzyme which acts both in decarboxylations and in transaminations. As indicated in the last column of the table we must now add to this list of vitamins which function in coenzymes, the compound pantothenic acid which proves to be a constituent of coenzyme A. As its name indicates, pantothenic acid is so widely distributed as to be considered a universal ingredient of living cells whether of microbial, plant, or animal origin. It is now found that the major part of this intracellular pantothenic acid occurs in combination as coenzyme A.

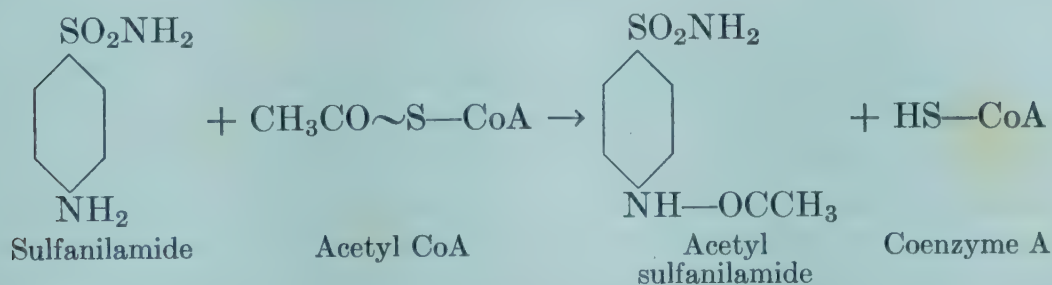
The compound has been obtained in a very nearly pure state and the formulation of its structure is virtually complete. It proves to follow the general pattern of several other compounds which act as coenzymes and to include adenylic acid linked through a pyrophosphate group to the vitamin part of the molecule. It is still uncertain whether the third phosphate group is attached to carbon 2 or 3 of the ribose.



As with other coenzymes, the activity of the molecule of coenzyme A is centered in one specific grouping, the terminal thiol group. Here in the presence of various enzyme systems and of ATP a bond is formed between the coenzyme and an acetyl group from a metabolite, giving the acetyl coenzyme A to which reference was made both in connection with oxidation of fatty acids and in the discussion of the Krebs oxidative cycle. One metabolite which readily acts as an acetyl donor is pyruvic acid which, in the presence of enzymes extracted from *Escherichia coli*, forms acetyl coenzyme A and formate. In subsequent formulations the free coenzyme will be represented as HS—CoA to emphasize the importance of the sulfhydryl group.



If sulfanilamide is added to this system its amino group is acetylated, and this is now believed to come about through transfer of the acetyl group from the acetylated coenzyme to the amine.

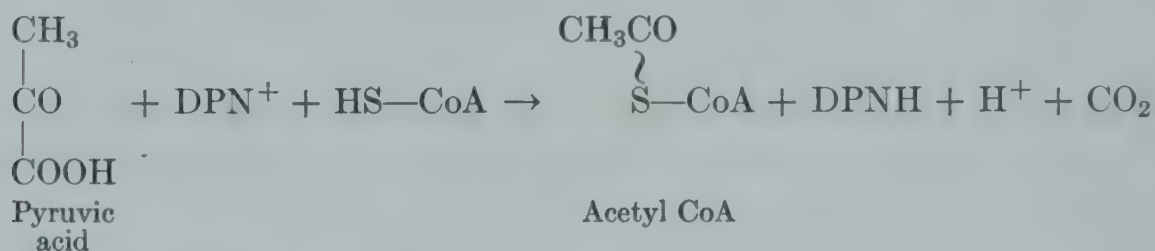


The acetylation of sulfanilamide proves to be but one of many reactions in which acetyl CoA takes part, and it is now generally accepted that in this compound active acetate has at last been identified and isolated. The bond between the acetyl group and the sulfur of the coenzyme is of the energy rich type, having a free energy of approximately 12,000 cal. per mole. The compound thus brings its own stored energy to any synthesis in which it takes part and so justifies its designation as "active." In 1951 Lynen in Munich isolated acetyl CoA from yeast, and highly purified samples of this compound have now been found to act as substrate in various synthetic procedures which had previously been ascribed to active acetate.

Formation of Acetyl Coenzyme A: If acetyl coenzyme A is the immediate source of the two-carbon unit used by the cell for synthesis, then the whole process in the course of which ordinary acetate is incorporated in a new molecule may be divided into two phases. In the first, acetyl groups are donated to the coenzyme: in the second, the activated acetyl coenzyme complex passes on its two-carbon unit to some acetyl acceptor. That this separation of the process into two steps has reality has been proved by the actual separation of the enzymes which catalyze the individual reac-

tions. In the presence of the "donating" enzyme acetyl CoA is formed, but unless the "transferring" enzyme is also present the acetyl group is not incorporated in a new molecule.

A good many substances in addition to acetate itself have proved to be acetyl donors. Among these compounds pyruvic acid is conspicuous, taking part in more than one type of enzymic reaction with coenzyme A. We have just seen that in the presence of bacterial enzymes the product, in addition to acetyl CoA, is formic acid. In the presence of the enzymes of the citric acid cycle, on the other hand, pyruvate reacts with coenzyme A in a simultaneous decarboxylation and dehydrogenation in which DPN^+ (CoI) acts as hydrogen acceptor. Under normal aerobic conditions the



reduced coenzyme I passes on to oxygen the hydrogens which it has acquired, while the acetyl groups may be used in several different ways.

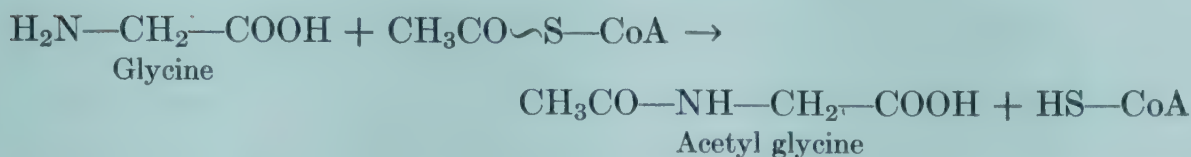
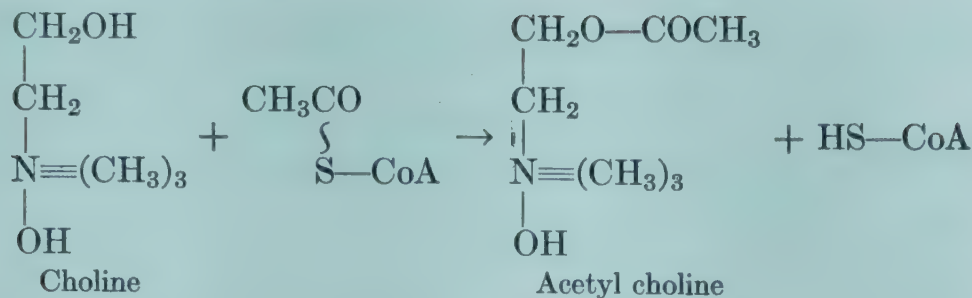
Other acetyl donors arise in the course of oxidation of fatty acids, of acetaldehyde, and of β -keto acids. The proteins also contribute to the metabolic pool of acetyl CoA, since several different amino acids are now known to yield pyruvate in the body either directly or indirectly. Thus many of the normal metabolic derivatives of the three primary foodstuffs serve as acetyl donors and thus, in conjunction with coenzyme A, give rise to active acetate.

For most of the known donors the actual course of the reaction by which two carbons are transferred to the coenzyme is not yet known. But Lipmann has very recently shown that with yeast or liver enzymes the transfer from acetate involves ATP in such a way that, in the course of the reaction, it loses its two terminal phosphate groups as free pyrophosphate. This undoubtedly requires more than one step, but may be summarized as follows:

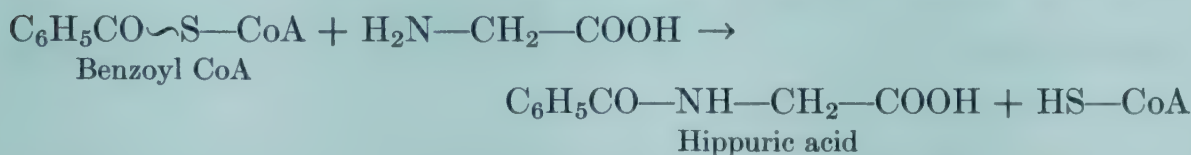


Synthetic Uses of Acetyl CoA: There are several different acetyl acceptors to which the acetylated coenzyme is now known to transfer its two-carbon unit, and it seems sure that many more acceptors will be found.

We have already noted the ability of acetyl CoA to acetylate sulfanilamide. Similar reactions give rise to acetyl choline and to acetylated

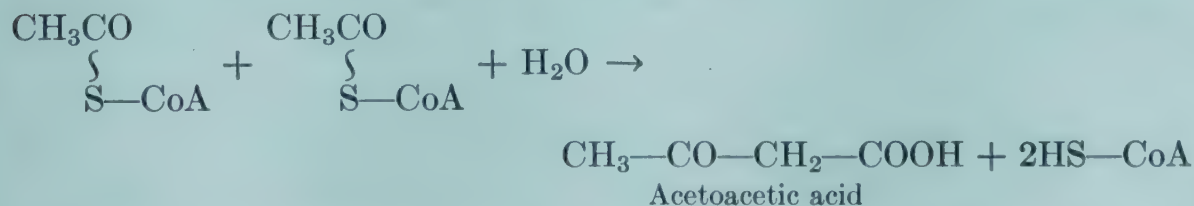


amino acids. In connection with the second reaction, it now appears that acids other than acetic acid may form acyl coenzyme complexes having properties similar to those of the acetyl compound. Benzoyl CoA for example would thus be the actual intermediate in the synthesis of hippuric acid. Furthermore, it appears that the active succinyl derivative



which is believed to be an intermediate in the biosynthesis of heme (p. 302) is also a coenzyme A complex.

Another type of reaction in which acetyl CoA donates its two-carbon unit is the one which results in synthesis of acetoacetate. This reaction, long associated with the idea of active acetate, takes place under the influence of pigeon liver extract. It is believed to involve two molecules of the acetylated coenzyme and one of water.



Another condensation reaction of acetyl CoA which has been fully authenticated is the one in which it reacts with oxalacetate to form citrate. This reaction was referred to briefly in connection with the citric acid cycle. The "condensing enzyme" which catalyzes citrate synthesis has recently been crystallized by Ochoa,¹ this being the first enzyme of the Krebs cycle to be obtained in crystalline form. The reaction which it catalyzes is the one by which pyruvate, transformed into the acetylated

¹ Dr. Severo Ochoa (1905-) was born and educated in Spain. He is now Professor of Pharmacology at the Medical College of New York University where he and his colleagues are in the forefront of present-day research in carbohydrate metabolism and cell oxidation.

bically send their carbons into the Krebs cycle by way of the acetylated coenzyme. Those amino acids which yield pyruvate, and there are several, are also metabolized by this pathway, while the ketogenic amino acids presumably yield acetoacetate by way of the same intermediate. We have seen that the β -oxidation of long chain fatty acids is believed to take place in such a way that each two-carbon unit is actually split out attached to coenzyme A and thus activated for any synthetic use for which the cell may need it. Probably the major part of the acetyl CoA formed in the cell transfers its two-carbon unit to oxalacetate, and thus sets it upon the pathway to complete oxidation by way of the Krebs cycle. Other acetylated coenzyme molecules are believed to be used for various other synthetic purposes. For example, two may condense as noted above to form acetoacetate. Or a single acetyl group may be donated to a pre-existing fatty acid to form the next higher homologue. Or, finally, a large number of molecules of acetyl CoA may condense with one another to form the carbon skeleton of a totally new acid. Since most of the reactions indicated in Figure 15.3 are reversible, it begins to be apparent why early experiments were able to show the "conversion" of one primary foodstuff to another.

ACTIVE FORMATE

There are many metabolic syntheses for which the cells are known to use either formate or a single carbon from the carbon chain of a more complex compound. This has given rise to the idea that in addition to active acetate there must be generated in the course of cell metabolism some active one-carbon unit which is referred to as "active formate." The experimental basis for this concept is as follows. If the carbons of glycine are labeled in such a way that both can be identified in metabolic products, it is found that the α -carbon is incorporated in several different molecules in which the carboxyl carbon is never found. Thus for example the α -carbon of glycine becomes the β -carbon of serine. Likewise the α -carbon of the amino acid is used in the synthesis of labile methyl groups which appear in the intact animal in choline or methionine. When it is demonstrated further that glycine can be replaced in both these reactions by formate but not by carbon dioxide or formaldehyde it is reasonable to assume that an intermediate in the transformation of the glycine carbon to either the β -carbon of serine or the methyl carbon of choline is formate or some closely related active fragment. These interrelationships are indicated in Figure 15.4, in which the labile carbons are printed in heavy type.

While there is as yet no definite proof of the way in which active formate is generated, there are certainly straws in the wind, and some of them at least point toward another one of the B vitamins, folic acid.

The establishment of a given compound as a "vitamin" is not as clear cut a matter as the list of compounds in Table 15-II might suggest. For example it may happen that in one laboratory a certain strain of bacteria is found to require small amounts of some factor which is finally separated and identified chemically. Perhaps later in another laboratory a substance which may be considered a derivative of the first proves necessary to the well-being of young chicks, while yet a third set of experiments with rats

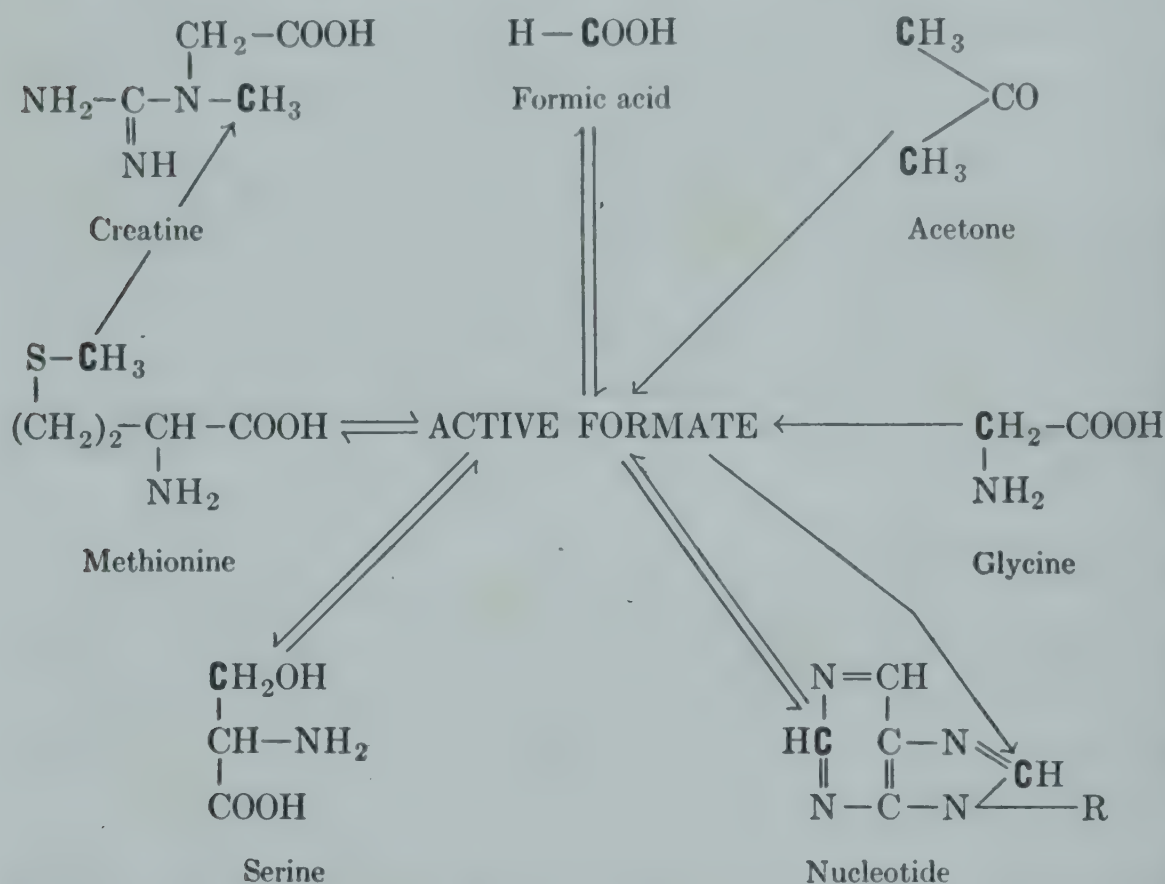
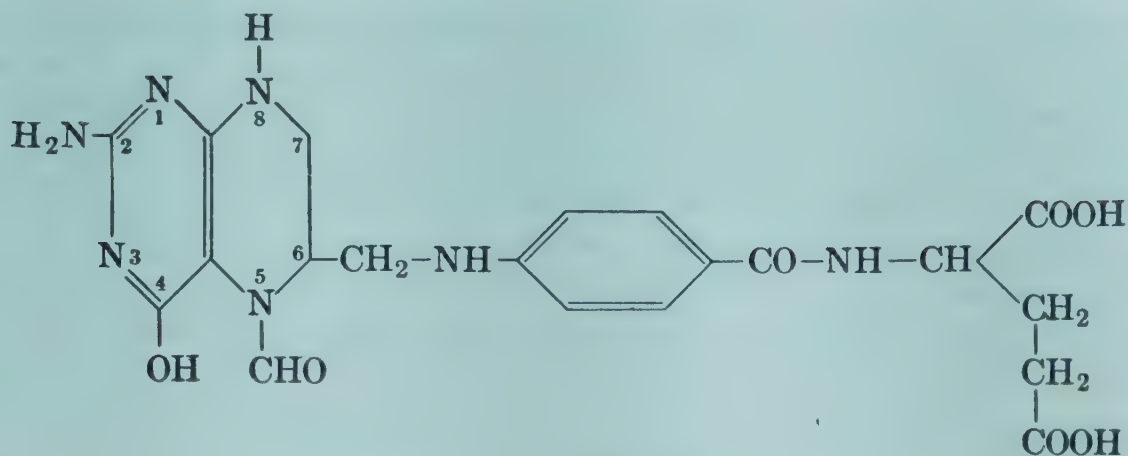


Figure 15.4. Interrelationships which point to the existence of an activated one-carbon intermediate.

may show that lack of a third related compound causes premature graying of the hair. Something of this sort happened with "folic acid." The unit which appears in several different factors required by various microorganisms is pteroylglutamic acid (PGA) which can often be transformed by a given species of bacteria into the specific factor which it requires. One of the metabolically active forms of PGA which has been variously called folinic acid, citrovorum factor and leucovorin, has now been synthesized and proves to be 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. The formyl group at position 5 in this compound is strikingly reminiscent of the acetyl group in acetyl CoA. Furthermore there is a growing body of evidence that folic acid is an essential factor for the reactions in which the one-carbon intermediate is believed to take part. Thus for example, rats deficient in folic acid incorporate less formate in serine than do paired rats given adequate amounts of the vitamin. Similarly the ability



of rat liver slices to incorporate formate into the purine ring, of which it can furnish carbons 2 and 8, is influenced by the availability of folic acid.

Here then we have (a) evidence for the existence of a one-carbon compound which is neither formate, formaldehyde, nor carbon dioxide, and which is therefore visualized as some activated molecule or complex. This is the more likely in that many of the reactions which incorporate "formate" in larger molecules require ATP. (b) We also have a vitamin which is apparently essential to the synthetic use of this activated formate, and which is known to occur as a formyl derivative. It is therefore tempting to speculate that this derivative is the active formate which in donating its formyl group to some cell constituent would free the vitamin for transfer of a second unit. Whether or not the cell actually does follow this familiar transfer pattern in using a one-carbon unit can only be determined by attempting to substitute for formate in cell-free enzyme systems the formyl derivative of pteroylglutamic acid. If this compound should prove active in synthesis, and other formyl compounds inactive, its importance in biosynthesis would have been established.

Fixation of Carbon Dioxide

By "fixation" of carbon dioxide is meant the use of its carbon for the synthesis of organic compounds. For many years it was believed that only solar radiation could furnish the energy needed for such endergonic syntheses, and that they were therefore the unique prerogative of green plants. This idea was first shown to be fallacious in work with bacteria. The so-called chemosynthetic bacteria use carbon dioxide as their sole source of carbon, but acquire the energy needed for its reduction not from sunlight but from oxidation of inorganic substances. More recently it has been found that even for heterotrophic organisms carbon dioxide is not simply a waste substance but is, on the contrary, a synthetic substrate with a positive role in cell metabolism. Thus the growth of yeasts, fungi, and bacteria in media which are otherwise adequate is prevented or

retarded if all their metabolic carbon dioxide is continually swept away by a stream of CO_2 -free air. Nor is the need for carbon dioxide limited to microorganisms. With various enzyme systems prepared from liver, kidney, or heart it has been shown that oxygen uptake is stimulated by the presence of carbon dioxide. Even more direct evidence has come from experiments with labeled carbon dioxide, which have led to incorporation of the carbon isotope in a number of different cell constituents, including glycogen and cell proteins. In a very recent study it was further found that injection of labeled bicarbonate in a cow gave rise to labeled lactose, casein, and fat in the milk. Thus the conclusion is inescapable that all known living organisms use carbon dioxide as a synthetic substrate. Its function in the self-nourishing autotrophs is obvious, for it furnishes the carbon for all their organic compounds. In the heterotrophs, whether animal or microbial, it is harder to understand why they use carbon dioxide as a synthetic substrate since they must oxidize some organic compounds in order to obtain energy for the synthesis of others.

A possible answer to this question is suggested by the fact that, as noted in Table 15-III, the carbon of carbon dioxide is nearly always incorporated in a carboxyl group. Furthermore, most of the acids in which it is fixed are familiar members of the citric acid cycle or of the glycolytic sequence. It may well be therefore that the use of carbon dioxide in synthesis assures to the cell an adequate supply of these acids which are essential to cell metabolism. Thus by expending various organic molecules which are present in abundance in the foodstuffs it could obtain both carbon and energy for synthesis of other compounds which play crucial roles in cell oxidation.

Not all of the compounds which ultimately acquire the carbon of carbon dioxide are primary fixation products. Some would clearly arise from organic precursors in the course of metabolic transformations. Thus the radioactive carbon which appears as carbon 6 of uric acid or as carbons 3 and 4 of glucose are undoubtedly many steps removed from the original carbon dioxide. A primary fixation reaction is one in which carbon dioxide itself reacts with some other carbon compound to form an organic carbon-to-carbon linkage. Whether in the cell there is one single fixation product or several is not known. Clearly the type of reaction by which carbon dioxide is likely to be transformed into a carboxyl group could well involve its addition to any one of a number of different compounds. This type of reaction is known as a *carboxylation*, and is in effect a reversal of such decarboxylations as those by which carbon dioxide is set free in the course of the citric acid cycle. In order to clarify the subsequent discussion of fixation reactions, it seems necessary at this point to bring together the information which is presently available about the enzymes which catalyze decarboxylations.

TABLE 15-III. COMPOUNDS IN WHICH CARBON DIOXIDE IS FIXED BY ANIMALS ^a

Name	Formula of Compound Analyzed ^b	Enzyme Source
Glycogen	CHO—CHOH— [*] CHOH— [*] CHOH—CHOH—CH ₂ OH Glucose	Liver slices
Proteins	HOOC— [*] CH(NH ₂)—CH ₂ —CH ₂ —COOH L-Glutamic acid	Rabbit liver or intact rat
	HOOC— [*] CH(NH ₂)—CH ₂ —COOH L-Aspartic acid	Rabbit liver
	HOOC—CH(NH ₂)—(CH ₂) ₃ —NH— [*] C(=NH)—NH ₂ L-Arginine	Rabbit liver or intact rat
Uric acid (excreted)	$ \begin{array}{c} \text{HN} - \overset{*}{\text{C}} = \text{O} \\ \quad \quad \quad \quad \text{H} \\ \text{OC} \quad \text{C} - \text{N} \quad \diagup \\ \quad \quad \quad \quad \diagdown \quad \text{CO} \\ \text{HN} - \text{C} - \text{N} \quad \diagup \\ \quad \quad \quad \quad \quad \text{H} \end{array} $	Intact pigeon
Pyruvic acid	CH ₃ —CO— [*] COOH	Pigeon liver
L-Lactic acid	CH ₃ —CHOH— [*] COOH	" "
Oxalacetic acid	HOOC—CO—CH ₂ — [*] COOH	" "
L-Malic acid	HOOC— [*] CHOH—CH ₂ — [*] COOH	" "
Fumaric acid	HOOC— [*] CH=CH—COOH	" "
α-Ketoglutaric acid	HOOC— [*] CO—CH ₂ —CH ₂ —COOH	" "

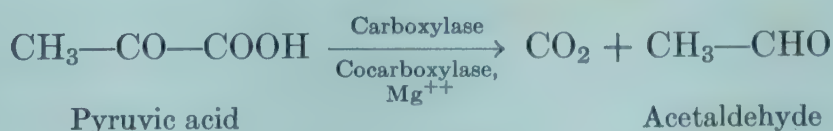
^a The data in this table are taken from H. A. Krebs, "Carbon Dioxide Fixation in Animal Tissues," in *Photosynthesis, No. V in the Symposia of the Society for Experimental Biology*, Academic, New York, 1951.

^b The results reported have all been obtained with radioactive HCO₃⁻ ion, and the fixed carbons are indicated by asterisks.

THE CARBOXYLASES

The enzymes which catalyze the splitting out of carbon dioxide from an organic acid have been called interchangeably *carboxylases* and *de-carboxylases*. The former term is perhaps preferable.

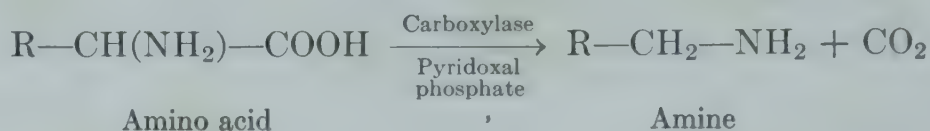
Neuberg discovered the first carboxylase in yeast in 1911. This was the pyruvic carboxylase which catalyzes the simple removal of carbon dioxide from pyruvate in the final steps of the fermentation cycle.



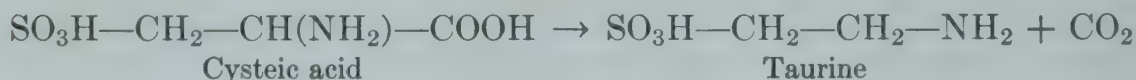
As was noted in the discussion of fermentation in Chapter 13, the enzyme requires the presence of magnesium ions and of cocarboxylase (diphosphothiamine). This reaction is, for all practical purposes, irreversible. The

carboxylase system has been found in yeast and in plants, but not in animal cells. When reference is made in the literature to "carboxylase," with no descriptive qualifications, it is this earliest known catalyst of this type which is meant. The crude enzyme preparations acted upon a number of different α -keto acids, but after purification proved to be very nearly specific for pyruvate.

Reference has already been made, in the chapter on Nitrogen Metabolism, to another simple type of decarboxylation by which amino acids are attacked by bacteria. This reaction leads to formation of highly toxic

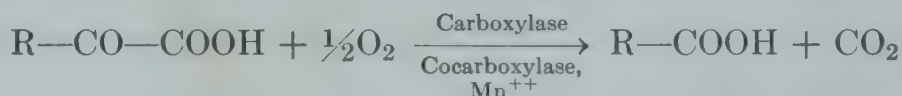


amines and diamines, and is rarely encountered in animal metabolism. However, both histidine and tyrosine carboxylases have been found in animal tissue, and a specific cysteic acid enzyme which catalyzes formation of taurine.



The coenzyme for the amino acid carboxylases is the same pyridoxal phosphate which acts as coenzyme in the transamination reaction.

Quite different from these simple decarboxylations are the enzymic *oxidative decarboxylations* for which magnesium or manganese ions, and diphosphothiamine are both required. Pyruvic acid undergoes this type of



decarboxylation as do some other α -keto acids. The equation represents the reactants and the end products, but the reaction is certainly far more complex than this would indicate. The metabolic importance of the oxidative decarboxylation of pyruvate was first brought out by the work of R. A. Peters at Oxford with vitamin B₁-deficient pigeons. He found that brain slices of such pigeons were unable to metabolize glucose past the pyruvate stage. When thiamine was added to the system it was promptly phosphorylated to cocarboxylase and the accumulated pyruvate then began to disappear with concomitant uptake of oxygen. The high concentrations of pyruvate in the tissues of human beings suffering from beri-beri indicate that one result of the B₁ deficiency is an inability to metabolize pyruvic acid, presumably for lack of the necessary coenzyme, cocarboxylase.

As shown in Table 15-IV, where the different classes of carboxylases are listed, the other acid which is known to undergo this same type of oxidative

TABLE 15-IV. REPRESENTATIVE DECARBOXYLATING ENZYMES

Substrate	Product in Addition to CO ₂	Source of Enzyme	Cofactors
I. Direct Carboxylases			
α -Keto acids	Corresponding aldehyde	Yeast, bacteria, plants	Mg ⁺⁺ Coccarboxylase
α -Amino acids ^a	Amines	Bacteria	Pyridoxal phosphate
II. Oxidative Carboxylases			
CH ₃ —CO—COOH Pyruvic acid	CH ₃ —COOH	Bacteria, muscle	Coccarboxylase
HOOC—CO—CH ₂ —CH ₂ —COOH α -Ketoglutaric acid	CH ₂ —COOH CH ₂ —COOH Succinic acid	Bacteria, heart and breast muscle	Coccarboxylase
III. β-Carboxylases			
H ₃ C—CO—CH ₂ —COOH Acetoacetic acid	CH ₃ —CO—CH ₃ Acetone	Liver, muscle, retina	Mn ⁺⁺ or Mg ⁺⁺
HOOC—CO—CH ₂ —COOH Oxalacetic acid	HOOC—CO—CH ₃ Pyruvic acid	Bacteria, plants	ATP and Mn ⁺⁺ or other divalent cation
CH ₂ —COOH CH—COOH CO—COOH Oxalosuccinic acid	CH ₂ —COOH CH ₂ CO—COOH α -Ketoglutaric acid	Muscle, plants	ATP and Mn ⁺⁺

^a Each of these enzymes is highly specific. A more complete list is given in Table 11.IX.

decarboxylation is α -ketoglutaric acid. This is the reaction by which succinic acid is formed in the citric acid cycle. The enzymes which catalyze these oxidative decarboxylations have been identified in animal tissues and in bacteria and in some plant tissues also.

Still a third type of decarboxylation is the one known as β -decarboxylation, because of the relation of the carboxyl group which is destroyed to the keto group in the molecule. The three acids of biological importance which are known to undergo this type of decarboxylation are acetoacetic, oxalacetic, and oxalosuccinic acids. The first loses carbon dioxide to form acetone and the second to yield pyruvic acid; oxalosuccinic acid as indicated in the table gives rise to α -ketoglutaric acid. The last three transformations require the presence of manganous or of magnesium ion, but do not require coccarboxylase.

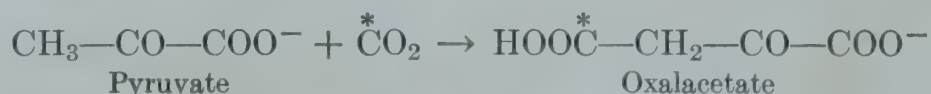
If the primary fixation reaction involves a simple reversal of decarboxylation, the specific catalyst or catalysts may well be found among these various carboxylases.

CARBON DIOXIDE FIXATION IN HETEROTROPHS

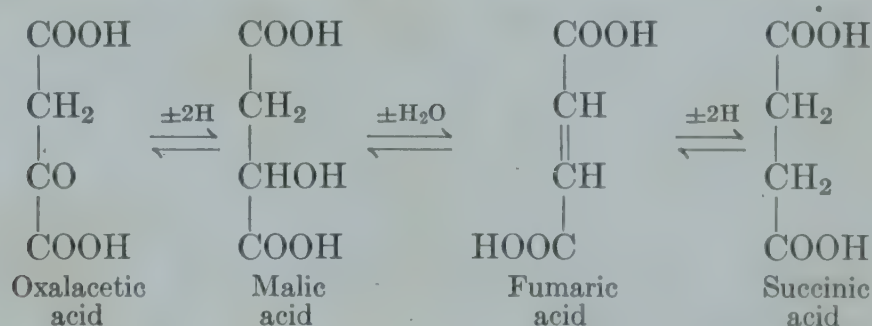
Recent studies of carbon dioxide fixation have made use of the enzymes of animals, of bacteria, and of green plants. These have thrown some light on the reactions by which autotrophic organisms make use of carbon dioxide, and on some very different reactions which are apparently involved in the fixation of carbon dioxide by heterotrophs. Among these latter two in particular are seriously considered as possible fixation mechanisms. These are the Wood-Werkman² reaction, and a reaction catalyzed by what is known as the "malic" enzyme.

The Wood-Werkman Reaction. The first evidence that carbon dioxide can be used for synthesis by heterotrophic cells was obtained by Wood and Werkman in 1936. The *Propionibacteria* constitute one of a mixed group of organisms which ferment various carbohydrates or glycerol with formation of propionic and other acids. Since carbon dioxide is evolved the amount of carbon in organic combination would be expected to fall somewhat during a fermentation. Wood and Werkman found however that in a medium containing bicarbonate ion the amount of organically bound carbon increased instead of decreasing. Furthermore they were able to show a clear stoichiometric relationship between the amount of carbon dioxide disappearing and the amount of succinic acid which accumulated. It has since been proved with labeled carbon dioxide that fermentation of glycerol does actually lead to incorporation of the marked carbon not only in succinic acid, but in propionic and acetic acids as well.

Wood and Werkman suggested that the primary reaction was probably a reversal of β -decarboxylation, leading to incorporation of carbon dioxide in oxalacetate.

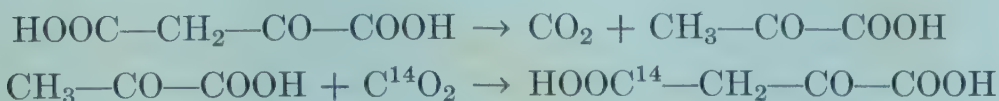


Since most cells contain the enzymes which catalyze the interconversions among the four dicarboxylic acids, this would account for the presence of isotopic carbon in succinate, as well as in other acids of the group.



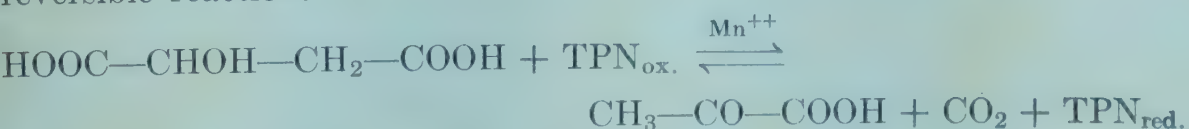
² Dr. Harland G. Wood (1907–) is now Professor of Biochemistry at Western Reserve University and Dr. C. H. Werkman (1893–) Professor of Bacteriology at Iowa State University. The experiments which linked their names to a reaction mechanism were done some years ago when both were at Iowa State.

In 1941 the enzyme responsible for decarboxylation of oxalacetate was found in bacterial extracts and it has since been extracted from pigeon liver and from various plant tissues. Although the equilibrium point of the enzymic reaction is far toward the side of decarboxylation it is possible with labeled carbon dioxide to demonstrate that it is a reversible reaction. This is done by incubating oxalacetate with the enzyme in the presence of labeled bicarbonate which acts as a reservoir of labeled carbon dioxide. The enzymic reaction is allowed to proceed until about half of the oxalacetate has been decarboxylated. At this point the reaction is stopped and the remaining oxalacetate is isolated. Since this compound proves to contain carbon isotope in the β -carboxyl group it is deduced that during the decarboxylation there is a continual simultaneous resynthesis of the oxalacetate. Because part of the available carbon dioxide is derived from the labeled bicarbonate, the carbon isotope becomes incorporated in molecules of the newly formed oxalacetate. The process of decarboxylation and resynthesis may thus involve different carbon dioxide molecules.



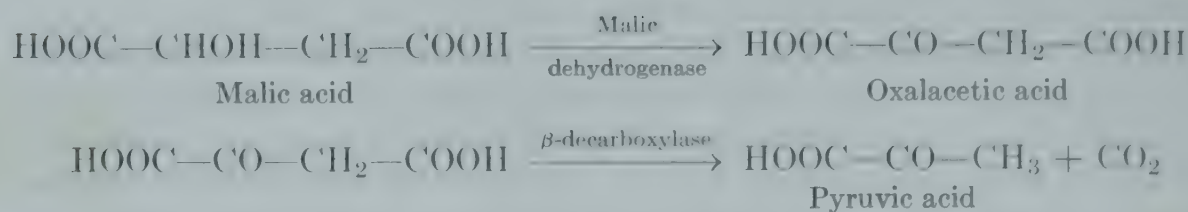
It should be noted that in such a reaction as this there is no net synthesis, that is, there is no net gain in the total amount of oxalacetate. This is often adduced as proof that this reaction cannot be responsible for carbon dioxide fixation. On the other hand it is also true that every biological preparation which has so far been found capable of fixing carbon dioxide has also proved able to decarboxylate oxalacetate, and it is hard to believe that this is a mere coincidence. Added to this is the fact that oxalacetate is metabolically closely related to many of the other acids in which carbon dioxide is incorporated enzymically and could easily give rise to them. There for the present the matter rests, with the likelihood that a β -carboxylation or something very like it may well be involved in carbon dioxide fixation.

The "Malic" Enzyme. Meantime Ochoa and his colleagues have discovered in pigeon liver an enzyme or enzyme system which catalyzes the reversible oxidative decarboxylation of the hydroxy acid malic acid. This system has also been extracted from plants and bacteria and has been found in a few animal tissues other than pigeon liver. It requires the presence of Mn^{++} and of TPN as hydrogen acceptor and catalyzes the following reversible reaction:



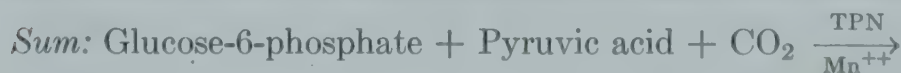
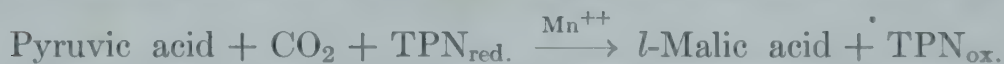
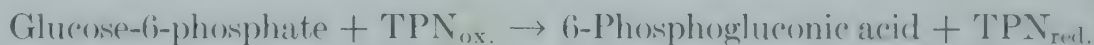
It was at first suggested that this reaction is the sum of two others the enzymes for which are well known: (a) oxidation of malate by malic

dehydrogenase, followed by (b) decarboxylation of the resulting oxalacetate.



But when highly purified samples of these two enzymes were allowed to act upon malic acid in the presence of the essential co-factors, it was found that the ready reversibility which characterizes the Ochoa system was entirely lacking. To distinguish this latter system from malic dehydrogenase it is referred to as the "malic" enzyme. When this enzyme is added to *l*-malic acid and TPN it is possible to follow spectroscopically the progressive, rapid reduction of the coenzyme as the acid is decarboxylated and oxidized in accordance with the first equation above; when the same enzyme is added to a mixture of pyruvate, bicarbonate, and reduced TPN the rapid disappearance of the absorption band of the reduced coenzyme indicates its oxidation in the course of the resynthesis of malate.

Although the equilibrium point of the "malic" system, like that of the Wood-Werkman system, lies far toward the products of decarboxylation, it has proved possible to couple it with another enzymic reaction and so to favor synthesis of malic acid by fixation of carbon dioxide. This is achieved by allowing the "malic" enzyme to act upon pyruvate and carbon dioxide in the presence of glucose-6-phosphate and its dehydrogenase. This latter enzyme, like the "malic" enzyme, specifically requires TPN as hydrogen acceptor, and the reaction has an equilibrium which greatly favors reduction of TPN⁺. By thus providing a continuous supply of reduced coenzyme the glucose system forces the "malic" reaction in the direction of fixation.



Thus the glucose phosphate furnishes both the hydrogens and the energy for the synthesis of *l*-malic acid. And in this reaction there is a clear net synthesis of new malic acid. Since malic acid is also a member of the citric acid cycle its synthesis from labeled carbon dioxide would account for the known distribution of isotopic carbon among the other acids of

the cycle. Furthermore by the reversal of the citric acid cycle it would give rise to labeled pyruvate, from which might be formed either alanine by transamination or glucose by reversal of the glycolytic sequence. By way of pyruvate also the carbon isotope could be incorporated in acetyl CoA, from which it would find its way into fatty acids or sterols.

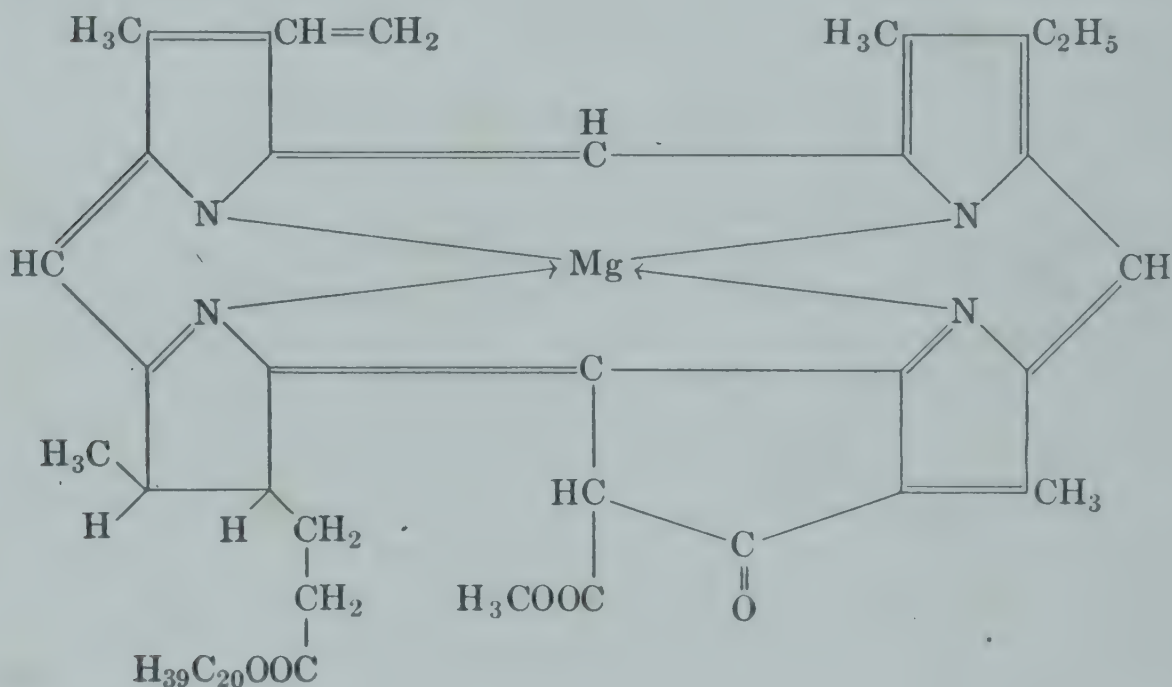
Obviously it is not at present possible to point to any one compound as the primary product of carbon dioxide fixation in heterotrophs. In spite of the fact that the two compounds which now seem the most likely candidates for this position are so closely related to each other chemically, neither one seems to be an intermediate in the synthesis of the other. At the same time the "malic" enzyme which has now been highly concentrated and purified proves always to catalyze not only the reversible "malic" reaction but also the decarboxylation of oxalacetate! And from one purification stage to the next its progressive increase in activity as a "malic" enzyme is paralleled by a corresponding increase in its activity as a β -carboxylase. Thus this enzyme takes its place with others which have been shown to exhibit a dual specificity, and which are therefore presumably equipped with two active centers. Perhaps this enzymic link between the two possible fixation mechanisms which we have been discussing will eventually lead to the elucidation of their role in carbon dioxide fixation.

FIXATION BY AUTOTROPHIC ORGANISMS

Although it has been known since the end of the eighteenth century that "fixed air" or carbon dioxide is incorporated by green plants in their own substance, no real attack on the chemistry of the process was possible before isotopic labels became available. Even now we know nothing of the individual enzymes concerned, nor of any single reaction in the sequence, since it has so far been necessary to work with intact cells in order to induce photosynthesis. Any analysis of the system into separate components makes it completely inactive in carbon dioxide fixation. Within these limitations however information has begun to accumulate leading to a better understanding of the parts played by light and by the chloroplasts, and of the path of carbon in photosynthesis.

The Chemistry of Chlorophyll. The catalyst chlorophyll which enables green plants to use the energy of sunlight for the reduction of carbon dioxide is a tetrapyrrole compound very like hemoglobin in structure. Willstätter, in whose laboratory it was first purified, separated the green coloring matter into chlorophyll *a* and chlorophyll *b* and proved that these two components are present in fairly constant ratio in the chloroplasts. The pigments are not isolated as conjugated proteins, although they probably occur in the plant in combination with protein. Chlorophyll *a* has the empirical formula $C_{55}H_{72}N_4O_5Mg$ and chlorophyll *b* differs only in having one more oxygen atom and two fewer hydrogens.

Decomposition of chlorophyll gives rise to substituted pyrroles very like those obtained from heme. Removal of the magnesium by treatment with acid leaves a waxy compound with no acidic groups, thus indicating that the metal is bound to nitrogen and not to an acidic group as a salt. As with heme, elucidation of the structure of the chlorophylls came as a result of work in many different laboratories, notably those of Willstätter and Hans Fischer in Munich and of Conant at Harvard. When the evidence was all in, chlorophyll *a* proved to be a magnesium porphyrin very closely allied to heme. As Fischer's formula indicates, it differs from heme in: (1) the saturation of Ring IV at positions 7 and 8; (2) esterification of one of the propionic acid groups with the long chain unsaturated alcohol, phytol;³ (3) the cyclization of the second propionic acid residue to give the five membered "carbocyclic" ring involving the γ - and the 6-positions; (4) esterification of the carboxyl group of this new ring with methyl alcohol; and (5) saturation of the vinyl group at carbon 4. Magnesium is held in the center of the flat ring exactly as iron is held in heme. Thus the red blood pigment and the green leaf pigment have the same fundamental structure.

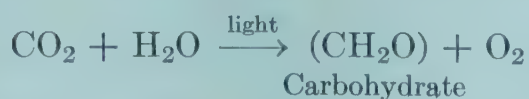


Chlorophyll *a*. (Fischer's structure)

Function of Chlorophyll in Photosynthesis. The broad outlines of the photosynthetic process were established in the course of about 30 years, beginning with Priestley's discovery that air which had been "injured" by burning a candle in it could be restored by vegetation. The importance

³ $(\text{CH}_3)_2\text{—CH—}(\text{CH}_2)_3\text{—}\underset{\text{CH}_3}{\text{CH}}\text{—}(\text{CH}_2)_3\text{—}\underset{\text{CH}_3}{\text{CH}}\text{—}(\text{CH}_2)_3\text{—}\underset{\text{CH}_3}{\text{C=CH—CH}_2\text{OH}}$.

of light, the participation of water, and the incorporation of the carbon dioxide in the plant fabric had all been recognized by the opening years of the nineteenth century. At that time the photosynthetic reaction might have been written:



On the assumption that the first step in photosynthesis is an interaction between water and carbon dioxide, various hypothetical intermediates have been suggested. The one for which there was at least a shadow of proof and which held the stage longest was the obvious one, formaldehyde. When Emil Fischer succeeded in preparing a dilute glucose solution by allowing formaldehyde to stand in the presence of barium hydroxide it seemed highly probable that a similar reaction took place in the plant and that formation of formaldehyde from water and carbon dioxide was followed by a sort of aldol condensation between six of these molecules to give the six-carbon carbohydrate chain. Partly for lack of any good alternative theory, formaldehyde continued to be the favored intermediate well into the twentieth century.

It is now known that even as a summary of events the simple formulation above is incorrect. This becomes apparent when the water provided for photosynthesis is labeled with O^{18} . The isotopic concentration of the oxygen evolved proves to be exactly like that of the water, showing that none of it is derived from the unlabeled carbon dioxide. Clearly then two molecules of water are involved for each molecule of carbon dioxide reduced and all of the free oxygen comes from the water.



That the photosynthetic process, here expressed as a single equation, actually involves two separate types of reaction has been known for many years. Of the two, only one requires sunlight; the subsequent reactions proceed equally well in light or darkness. Thus green cells which are illuminated in the absence of carbon dioxide must undergo some undefined photochemical change, for when they are later brought into contact with carbon dioxide, even in the dark, they are able to bring about its fixation. Further analysis of the photosynthetic process therefore requires examination of the light reaction and of those reactions which take place in the dark.

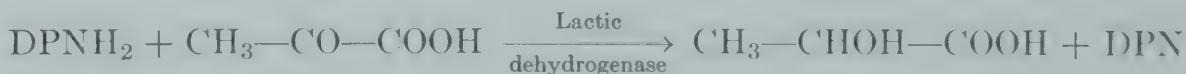
It will be recalled that chlorophyll occurs in green leaves in the small bodies known as chloroplasts, of which there may be as many as 100 in a single leaf cell. Within the light-colored stroma of the chloroplasts are the minute, dark *grana* in which the chlorophyll is concentrated, possibly

as a protein-chlorophyll complex, though there is some evidence that the complex contains lipid material as well. If green leaves are finely ground it is possible to separate from the tissue residue a fluid which contains whole and broken chloroplasts. This green fluid is not able to fix carbon dioxide, but if it is illuminated it is still capable of setting free oxygen if hydrogen acceptors are available. This reaction in which chlorophyll catalyzes the evolution of oxygen from water and the reduction of some hydrogen acceptor is known as the *Hill reaction*. In the expressed cell juices there may be natural acceptors, or artificial ones such as quinone or ferricyanide may be added to the medium. Thus Warburg has shown that the chloroplast substance will reduce quinone to hydroquinone in the light, with a corresponding evolution of oxygen. This is believed to indicate that the light-induced step in photosynthesis, for which chlorophyll acts as catalyst, is a splitting of water. In this process oxygen is set free, and the hydrogen becomes attached to some compound in the leaf cell which normally uses it to begin the reduction of carbon dioxide or of some derivative of carbon dioxide. When no carbon dioxide is available at the moment of illumination, the reducing compound apparently accumulates and can act upon carbon dioxide later in the dark. In these terms the essential photochemical reaction is the generation of reducing power.

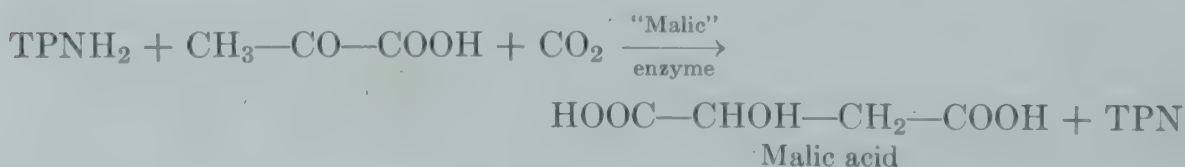
The nature of the hypothetical hydrogen acceptor is still obscure, but there is one bit of recent evidence which may have some bearing on the problem. Ochoa has found that suspensions of the green grana of spinach leaves catalyze the photochemical reduction of both pyridine nucleotides, Co I and Co II. This reduction he has been able to couple with other reactions and so to bring about syntheses which take place only if both the grana and light are available. On this basis it is possible that the first light reaction consists of a reduction of one or both of the familiar coenzymes by water.



In the presence of a DPN-specific dehydrogenase Ochoa has been able to prove a light-induced synthesis of lactic acid from pyruvic.



Furthermore, by coupling with the TPN-specific "malic" system, he has brought about fixation of carbon dioxide in malic acid.



If it should prove that the pyridine nucleotides really play a major part in photosynthesis it would be a delightful and striking example of the unified metabolic pattern which seems to characterize even the most diverse organisms.

The Path of Carbon in Photosynthesis. While one group of investigators has focused attention upon the photochemical generation of reducing power, others have concerned themselves with the chemical reactions in which carbon to carbon bonds are formed in green plants. This second type of experiment only became possible with the advent of isotopic carbon and of effective analytical procedures for the separation and identification of small amounts of very similar compounds. The first studies were carried out when the only available carbon isotope was the short-lived C^{11} , but since 1948 the carbon dioxide used for this type of photosynthetic investigation has been labeled with the long-lived radioactive C^{14} .

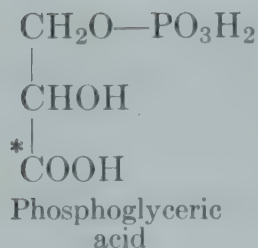
The outstanding recent contributions to the elucidation of the path of carbon in photosynthesis have come from Calvin's ⁴ group in California and Gaffron's ⁵ group in Chicago. In both laboratories the experiments are designed to allow identification of the earliest product of photosynthesis. For this purpose green algae are allowed to assimilate labeled carbon dioxide for brief periods and are then killed and extracted. In the Calvin experiments, for example, the algae undergo a brief period of "pre-illumination" and are then allowed to fix $C^{14}O_2$ in the dark. The soluble compounds are extracted from the plants at the end of the fixation period, separated by two-dimensional paper chromatography, and allowed to identify themselves by forming a radioautograph on a photographic film.

It was found that after ninety seconds of dark fixation fifteen different compounds had acquired the radioactive label. In thirty seconds C^{14} was incorporated in eight different substances, among them the five acids, glycolic, malic, aspartic, phosphopyruvic, and phosphoglyceric acids. The other three substances which were identified were a triose phosphate, hexose monophosphate, and hexose diphosphate. Most of these compounds are familiar intermediates in glycolysis or in the citric acid cycle, and they therefore suggest that photosynthesis may turn out to be in detail as well as in general, a reversal of respiration.

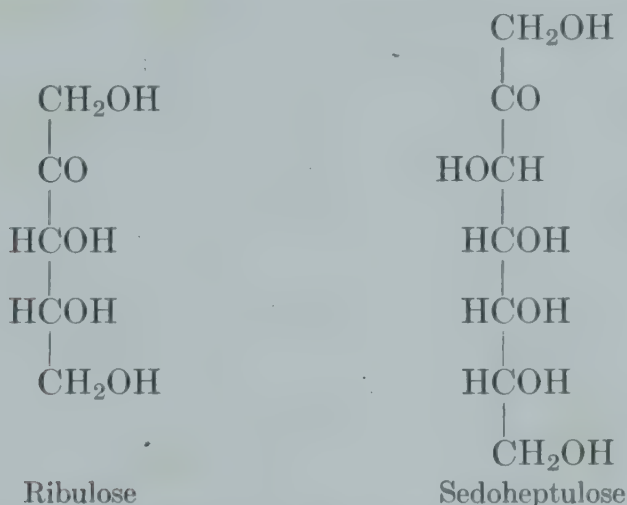
Shortening the period of dark fixation to five seconds resulted in confining the isotope to four or five compounds, with 65 per cent of it in the carboxyl group of phosphoglyceric acid.

⁴ Professor Melvin Calvin (1911-) is Director of the Biochemical Division of the Radiation Laboratory at the University of California in Berkeley. His interest in the mechanism of organic reactions is being applied specifically to the mechanism of photosynthesis.

⁵ Hans Gaffron (1902-) was born in Peru and educated in Berlin where he carried on his early biochemical research. Since 1939 he has been at the University of Chicago where he is now Associate Professor of Biochemistry. He has been chiefly interested in plant physiology, in the metabolism of the purple bacteria, and in photochemistry in general.



This observation has, after some preliminary disagreement, been confirmed in Gaffron's laboratory so that the problem of photosynthesis may now be posed in two related questions: (1) by what chemical reaction is carbon dioxide incorporated in the carboxyl group of phosphoglyceric acid, and (2) what chemical reactions lead from this compound to glucose? For neither of these questions is there as yet any generally acceptable answer. It is assumed that the labeled carboxyl group is the result of a direct carboxylation, and it was at first believed that some sort of cycle must continually generate a two-carbon compound which, in accepting carbon dioxide, would give rise to glyceric acid. That such a cycle would not prove simple became evident when there were identified among the products of photosynthesis both the five-carbon keto sugar, ribulose, and the seven-carbon ketose, sedoheptulose.



As a result of very recent work on the exact distribution of radioactive carbon in the compounds formed in the early stages of photosynthesis, Calvin's group now suggests that the acceptor of carbon dioxide is not a two-carbon compound, but the diphosphate of the ketopentose, ribulose. This compound is believed to react with carbon dioxide and water to yield two molecules of phosphoglyceric acid, perhaps with the intermediate formation of a β -keto acid.

Figure 15.5, in which all the phosphate groups have been omitted for the sake of simplicity, shows in somewhat simplified form the cycle now proposed by Calvin and his colleagues. The reducing power which is generated in the light reaction is indicated by $[\text{H}]$, and the compounds are

designated in terms of the number of carbon atoms they contain. The cycle consists essentially of six steps, of which the one involving carbon dioxide may be considered to be the first.

1. Carbon dioxide, reacting with a molecule of ribulose diphosphate and one of water, gives rise to two molecules of phosphoglyceric acid, perhaps with intermediate formation of a six-carbon acid before a hydrolytic step.

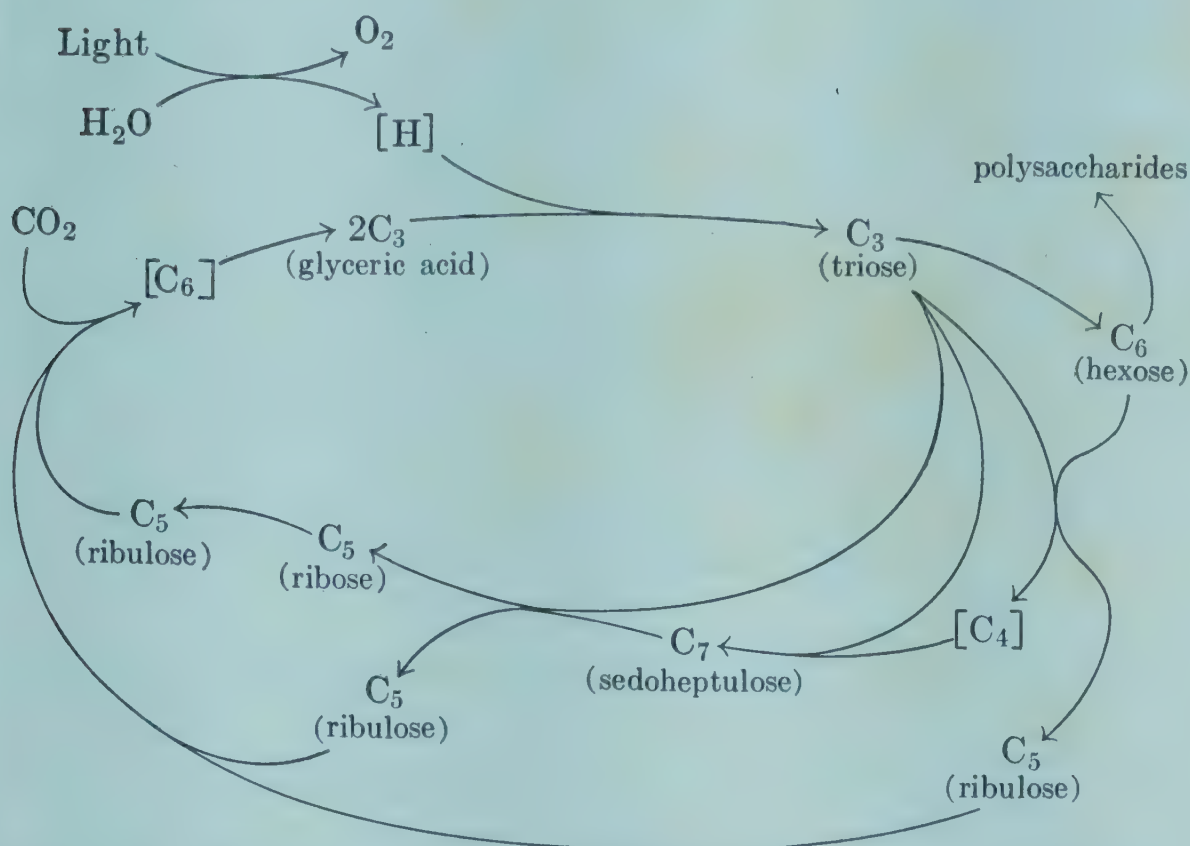


Figure 15.5. Proposed cycle for carbon reduction in photosynthesis. (Adapted from J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson, and M. Calvin, *J.A.C.S.*, 76:1760, 1954.)

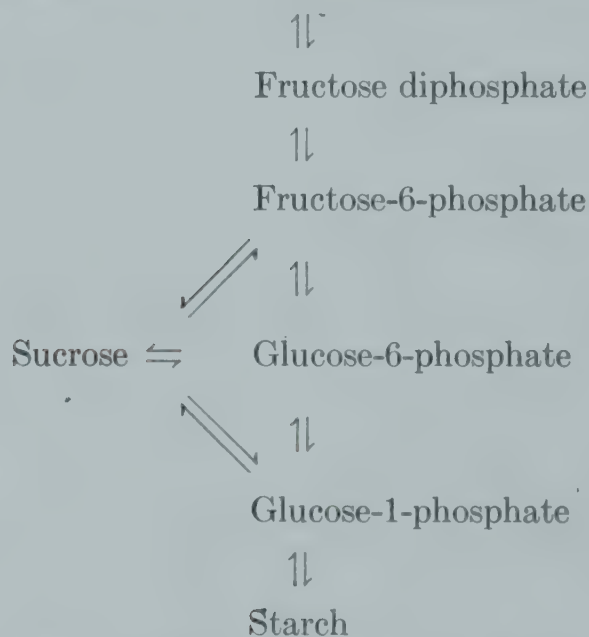
2. The reducing power generated in the light reaction reduces the phosphoglyceric acid to phosphotriose.
3. (a) Two phosphotriose molecules may condense to form hexose phosphate, which may in turn give rise to polysaccharides.
(b) Alternatively the newly formed hexose phosphate may react with another molecule of triose phosphate to yield one molecule of ribulose phosphate and one of an undefined tetrose phosphate (C₄).
4. The C₄ compound, reacting with another molecule of triose would account for the seven-carbon sedoheptulose phosphate.
5. Sedoheptulose phosphate, reacting with still another triose phosphate molecule, yields two five-carbon sugars, ribose and ribulose phosphates.
6. With the transformation of the aldose, ribose, into the ketose, ribulose, the cycle is complete. Three molecules of ribulose phosphate and one

of triose phosphate have been synthesized from an original three molecules of ribulose phosphate and three of carbon dioxide.

The steps which lie between phosphoglycerate and glucose are also undefined, though there are two bits of evidence which suggest that they may involve the same transformations as those which are used by animal tissues for similar syntheses. It will be recalled that liver enzymes catalyze reactions in which labeled carbon dioxide is incorporated into glycogen. Analysis of this radioactive glycogen has shown that the first carbons to be labeled are carbons 3 and 4 of the glucose moiety, as if the six-carbon molecule had been formed by condensation of two triose molecules each labeled in a terminal carbon. When photosynthesis is allowed to proceed until part of the carbon isotope has been incorporated in carbohydrates it is found that in this process also the first carbons to be labeled are carbons 3 and 4 of the hexose molecules. This is interpreted to mean that the single-labeled three-carbon molecules of phosphoglyceric acid or of some closely related derivative interact in such a way that a link is formed between the two labeled carbons. Such a reaction would be exactly analogous to the one which is believed to take place in liver tissue.

SUGGESTED PATHWAY OF CARBOHYDRATE SYNTHESIS IN PLANTS

Phosphoglycerate \rightarrow Triose phosphate

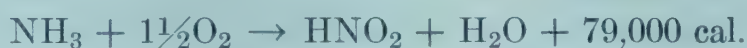


If the mechanism of photosynthetic assimilation of carbon dioxide involves reactions which reverse the glycolytic cycle, the labeled carbon should appear in the various members of the cycle in the reverse sequence. For example it should appear in fructose before it appears in glucose, since fructose diphosphate appears later in the glycolytic sequence. Present evidence indicates that this is indeed true, for when labeled sucrose first appears in photosynthesizing algae the fructose

moiety contains more of the label than does the glucose, though later of course both will have acquired the isotope. This would be explained if the formation of sucrose depended, as indicated in the chart, on a reversal of glycolysis to the point at which both glucose and fructose were available.

CHEMOSYNTHESIS

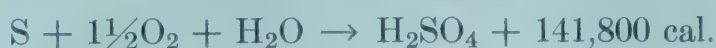
Among the autotrophic bacteria a few are equipped with pigments known as bacteriochlorophylls by means of which they are able to draw upon solar energy for synthetic purposes. These are the various purple and green bacteria which, although they live anaerobically, meet their synthetic needs in essentially the same way as do the higher plants. In addition there are a number of aerobic autotrophic organisms which derive their energy from the oxidation of inorganic substances. This group includes the soil bacteria which oxidize ammonia or nitrites to the nitrate level, as well as a number of organisms which oxidize sulfur or sulfur compounds. All of these so-called chemosynthetic bacteria develop in media which are entirely devoid of organic carbon, and indeed for some of them organic compounds are actually toxic. The energy which they use in synthesis they obtain from strongly exergonic oxidations. Thus the *Nitrosomonas* which oxidize soil ammonia to the nitrite level acquire in the process 79,000 cal. per mole of ammonia.



The further oxidation of nitrites to nitrates by which the nitrogen is made available as a plant food is brought about by *Nitrobacter*.

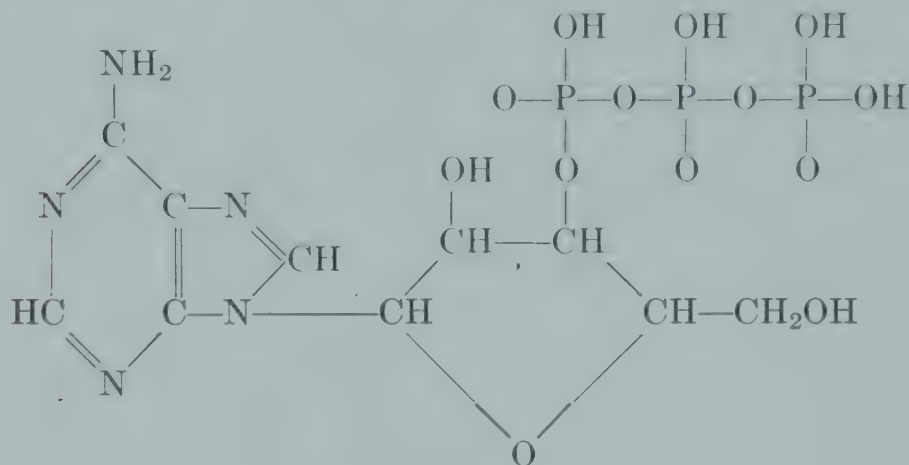


A third organism of this type, the *Thiobacillus thiooxydans*, acquires its energy by oxidation of elementary sulfur to sulfuric acid and manages to flourish at a pH which is lethal for most living cells.



As might be expected, very little is known about the mechanism by which the energy made available in these oxidations is transformed into the chemical energy of organic compounds. Yet what little is known of the process indicates that these minute bodies depend upon much the same procedures and mechanisms as are characteristic of multicellular organisms. For example it has been found that the chemosynthetic process, like the photosynthetic one, can be analyzed into two separate steps. If carbon dioxide is excluded, the sulfur bacteria can still oxidize sulfur or hydrogen sulfide, and this is achieved in such a way that when carbon dioxide later becomes available it is rapidly assimilated, as in the corresponding dark reaction in plants. Furthermore, during the oxidative phase inorganic phosphate disappears and organic phosphate is formed.

Later when the carbon dioxide is being built into organic compounds, there is a simultaneous liberation of inorganic phosphate. The organic phosphate compound which has this transitory existence between the two stages of chemosynthesis has been isolated and identified, and proves to be a form of the ubiquitous ATP, differing from the more common substance only in having the pyrophosphate linked to carbon 3 of the



Adenosine-3-triphosphate

ribose instead of carbon 5. Here then at a very different evolutionary level from that of animals or of the higher plants, is a familiar biosynthetic mechanism. The energy of oxidation is stored or trapped as high energy phosphate bond energy and this in turn is transformed into the energy of organic compounds by allowing the phosphate to drop back to the inorganic level.

Nor does the comparison end here. The *Thiobacillus* lives at a pH which is always very low and may fall to zero. Under these circumstances it might have been expected to exhibit a very special metabolism, and to synthesize products which would be unique. But this proves not to be true. These organisms elaborate just such a series of phosphate esters, including hexose mono- and di-phosphates, phosphoglyceric acid, and phosphopyruvic acid, as would indicate that their carbohydrate metabolism follows familiar pathways. Furthermore, in addition to the usual structural and food units these bacteria elaborate thiamine, riboflavin, nicotinic and pantothenic acids, biotin, and coenzyme I. Thus they synthesize and presumably use a very nearly complete battery of the B vitamins. This would certainly indicate that in their metabolic processes they also find use for the same coenzymes which function in the cells of animals and of plants.

It would of course be rash to generalize even about microorganisms on the basis of results with one specific strain. This is true not only because of the great number and variety of bacterial species, but because these small organisms possess a staggering ability to adapt themselves to new

circumstances by developing new metabolic capabilities. Nonetheless, there do seem to be certain fundamental biochemical procedures which are universally distributed and which with minor adaptations serve the needs of the most diverse living forms. Thus the reactions and enzymes of the glycolytic sequence appear again and again, either leading to degradation or to synthesis. Energy is trapped and stored in phosphate bonds; synthesis is achieved through the mediation of ATP or of coenzyme A. Many of the essential food factors which we call vitamins in speaking of animal nutrition are equally essential to the growth of heterotrophic bacteria. The vitamins which animals must obtain from plants presumably serve similar metabolic functions in the plant which elaborates them. Thus from the biochemical studies of the past and the present, which often seem to deal with such a confusing multiplicity of phenomena, there is beginning to emerge a pattern. It is a complex pattern, only dimly apprehended as yet, but a pattern to stir the imagination as it must always be stirred by any manifestation of beauty.

Suggestions for Further Reading

ACTIVE ACETATE

Two reviews in the *Ann. Rev. Biochem.*, 21, 1952, bring together the results in this area:

BLOCH, K., "Interrelationships of Lipid and Carbohydrate Metabolism."

OCHOA, S., and STERN, J. R., "Carbohydrate Metabolism."

BRADY, R. O., and GURIN, S., "Biosynthesis of Fatty Acids by Cell-Free or Water-Soluble Enzymes," *J.B.C.*, 199:421, 1952.

CHOU, T. C., and LIPMANN, F., "Separation of Acetyl Transfer Enzymes in Pigeon Liver Extract," *J.B.C.*, 196:89, 1952.

JONES, M. E., LIPMANN, F., HILZ, H., and LYNEN, F., "On the Enzymatic Mechanism of Coenzyme A Acetylation with Adenosine Triphosphate and Acetate," *J.A.C.S.*, 75:3285, 1953.

LITTLEFIELD, J. W., and SANADI, D. R., "Role of Coenzyme A and DPN in Oxidation of Pyruvate," *J.B.C.*, 199:65, 1952.

LIPMANN, F., JONES, M. E., BLACK, S., and FLYNN, R. M., "Enzymic Pyrophosphorylation of Coenzyme A by Adenosinetriphosphate," *J.A.C.S.*, 74:2384, 1952.

LYNEN, F., REICHERT, E., and RUEFF, L., "Active Acetate, Its Isolation from Yeast and Its Chemical Nature," *Ann.*, 574:1, 1951.

NOVELLI, G. D., SCHMETZ, F. J., JR., and KAPLAN, N. O., "Enzymatic Degradation and Resynthesis of Coenzyme A," *J.B.C.*, 206:533, 1954.

ACTIVE FORMATE

In the *Ann. Rev. Biochem.*, 21, 1952, the experimental data on which this concept is based are brought together and discussed in:

TARVER, H., "Metabolism of Amino Acids and Proteins," pp. 309-10.

WELCH, A. D., and NICHOL, C. A., "Water Soluble Vitamins," pp. 655-665.

- ARNSTEIN, H. R. V., "Biosynthesis of Choline Methyl Groups," *B.J.*, 48:27, 1951.
 GREENBERG, G. R., "A Formylation Cofactor," *J.A.C.S.*, 76:1458, 1954.

CARBON DIOXIDE FIXATION

"Carbon Dioxide Fixation and Photosynthesis," *Symposium No. V of the Society for Experimental Biology*, Academic, New York, 1951.

This volume contains a number of summarizing papers by chemists, botanists, and biochemists and provides excellent bibliographies on many different phases of the subject.

- UTTER, M. F., and WOOD, H. G., "Mechanisms of Fixation of Carbon Dioxide by Heterotrophs and Autotrophs," *Adv. in Enzym.*, 12:41, 1951.
 BENSON, A. A., KAWAUCHI, S., HAYES, P., and CALVIN, M., "Path of Carbon in Photosynthesis, XVI," *J.A.C.S.*, 74:4477, 1952.
 BASSHAM, J. A., BENSON, A. A., KAY, L. D., HARRIS, A. Z., WILSON, A. T., and CALVIN, M., "Cyclic Regeneration of Carbon Dioxide Acceptor," *J.A.C.S.*, 76:1760, 1954.
 CALVIN, M., "The Path of Carbon in Photosynthesis," *Chem. and Eng. News*, 31:1622, 1953.
 CALVIN, M., "The Quantum Conversion in Photosynthesis," *Chem. and Eng. News*, 31:1735, 1953.
 KLEIBER, M., SMITH, A. H., and BLACK, A. L., "Carbonate as Precursor of Milk Constituents in the . . . Cow," *J.B.C.*, 195:707, 1952.
 OCHOA, S., "Biological Mechanisms of Carboxylation and Decarboxylation," *Physiol. Revs.*, 31:56, 1951.
 VENNESLAND, B., CEITHAML, J., and GOLLUB, M. C., "Fixation of Carbon Dioxide in a Plant Tricarboxylic Acid System," *J.B.C.*, 171:445, 1947.
 VISHNIAC, W., and OCHOA, S., "Fixation of Carbon Dioxide Coupled to Photochemical Reduction of Pyridine Nucleotides," *J.B.C.*, 195:75, 1952.

CHLOROPHYLL

FISCHER, H., "Chlorophyll," *Chem. Revs.*, 20:41, 1937.

This is an excellent summary paper written for the Harvard Tercentenary in 1936.

STEELE, C. C., "Chlorophyll," in Gilman, H. (ed.), *Organic Chemistry: An Advanced Treatise*, Wiley, New York, 1943.

Study Questions

1. List with their formulas several compounds which have high energy phosphate bonds; one in which a high energy bond is a carbon-to-sulfur linkage.
2. Which are the high energy bonds in ATP? Under what conditions can such a bond be formed? List several ways in which the energy of such a bond is used by the cell.
3. Write the formula of the compound which is described as "active acetate."
4. Indicate three different synthetic reactions of active acetate.

5. What type of compound is coenzyme A? What is its functional group?
6. List the vitamins which are known to be part of coenzyme molecules, and for each indicate the enzyme or enzymes for which it acts as coenzyme.
7. What is known of the mechanism of the reaction in which the acetyl group of acetic acid is donated to coenzyme A? List several other acetyl donors.
8. List several reactions in which the acetyl group of acetyl coenzyme A is accepted by other molecules. Which of these reactions is of special importance in the oxidative mechanism of the cell?
9. What is the experimental evidence for the existence of "active formate"?
10. Define "fixation" of carbon dioxide. What is a primary fixation product?
11. What is the experimental evidence for the synthetic use of carbon dioxide by heterotrophic organisms?
12. What reaction is catalyzed by carboxylases? What are the different types of carboxylases?
13. What is cocarboxylase and with what specific enzymes does it act?
14. What is the Wood-Werkman reaction? the Hill reaction?
15. What is the "malic" enzyme and what is its importance? Write equations to show how the "malic" reaction may be coupled with oxidation of glucose-6-phosphate.
16. What reaction is believed to be catalyzed by chlorophyll in the light? How is this reaction related to carbon dioxide fixation?
17. What is the earliest product of carbon dioxide fixation which has so far been identified? By what type of reaction is it believed to arise?
18. Write the formulas for ribulose and for sedoheptulose. How are these compounds believed to be related to photosynthesis?
19. What evidence is there that beyond the three-carbon stage photosynthesis is a reversal of glycolysis?
20. What is meant by "chemosynthesis"? Write equations for two different reactions which furnish the energy for this type of synthesis and indicate the organisms which bring them about.

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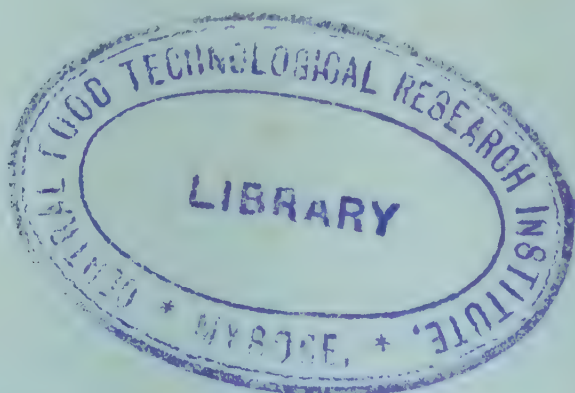
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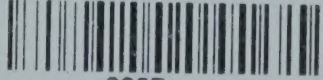
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